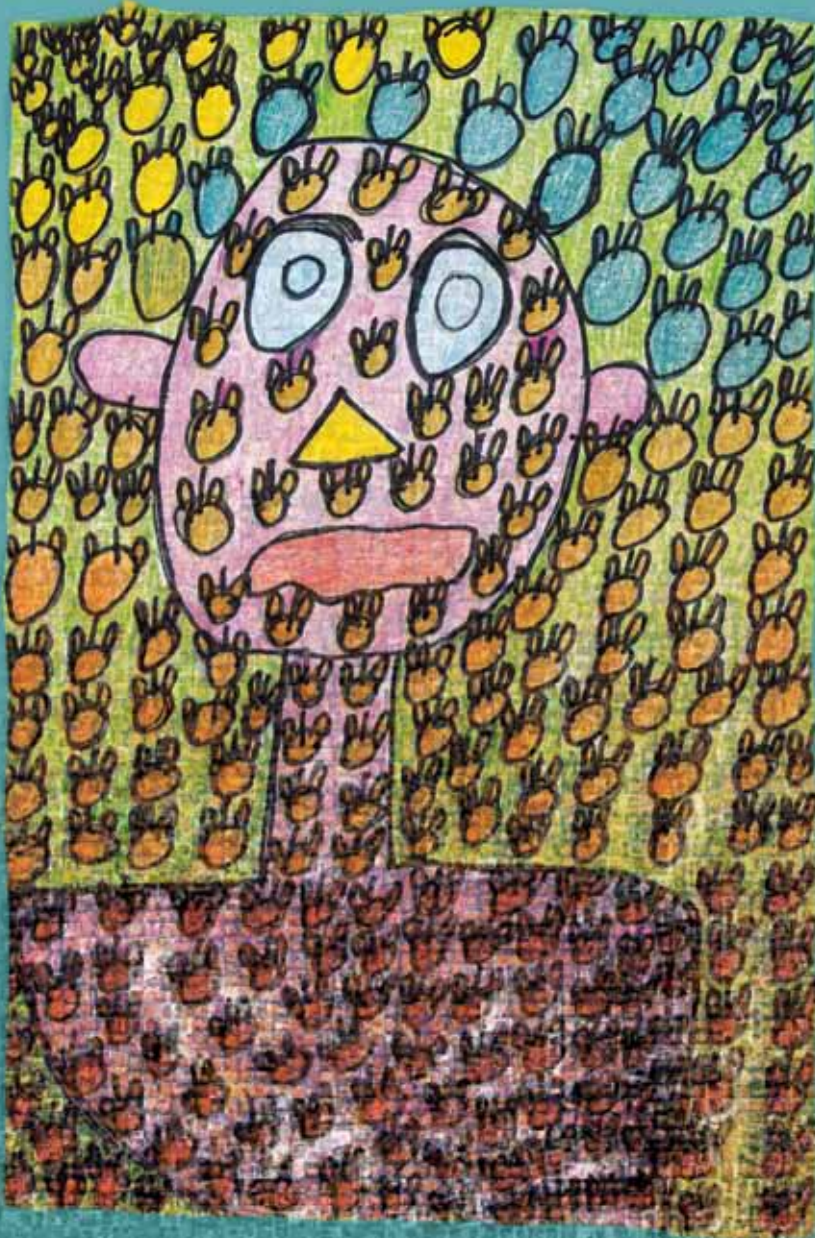


# Making Headway with the Molecular and Clinical Definition of Rare Genetic Disorders with Intellectual Disability



Marjolein H Willemsen

# **Making Headway with the Molecular and Clinical Definition of Rare Genetic Disorders with Intellectual Disability**

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**Marjolein Hanna Willemsen**  
geboren op 28 februari 1981  
te Nijmegen

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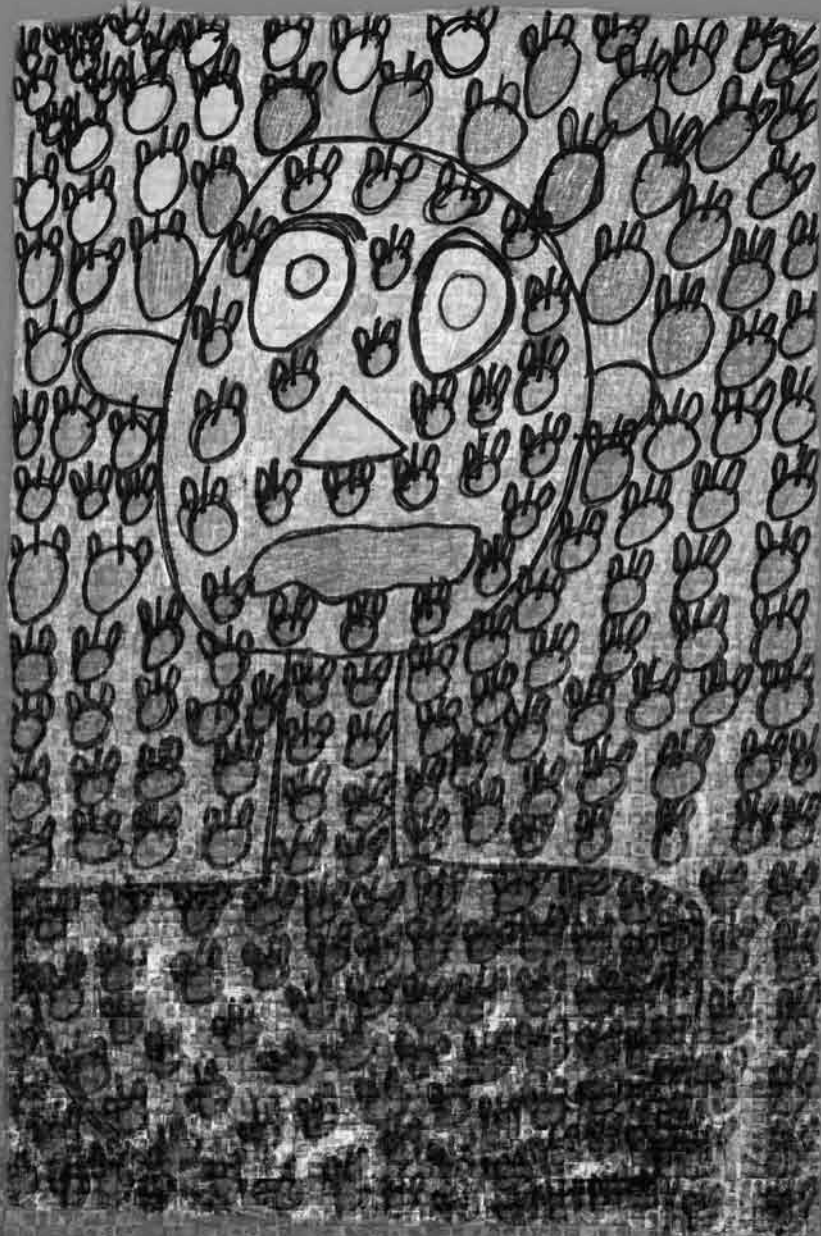
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## List of abbreviations

AAIDD	American Association on Intellectual and Developmental Disabilities
AD	Autosomal dominant
ADHD	Attention Deficit Hyperactivity Disorder
AR	Autosomal recessive
ASD	Autism spectrum disorder
ATRX	Alpha-thalassemia/mental retardation syndrome
CA	Congenital anomaly
CD	Cognitive disorders
CMT	Charcot-Marie-Tooth
CNV	Copy number variation
CNS	Central nervous system
CT	Computer tomography
DD	Developmental delay
DECIPHER	DatabasE of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources
DGV	Database of Genomic Variants
ECARUCA	European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations
EEG	Electroencephalogram
FISH	Fluorescent <i>in situ</i> hybridization
HMM	Hidden Markov model
HPO	Human Phenotype Ontology
ICD	International Classification of Diseases
ID	Intellectual disability
IQ	Intelligence quotient
IUGR	Intra-uterine growth retardation
kb	kilobase (thousand base pairs)
KIF	Kinesin family
KS	Kleefstra Syndrome
Mb	Megabase (million base pairs)
MCA	Multiple congenital anomalies
MLPA	Multiplex Ligation Probe Amplification
MRI	Magnetic resonance imaging
NGS	Next generation sequencing
OMIM	Online Mendelian Inheritance in Man
SNP	Single Nucleotide Polymorphism
UPD	Uniparental disomy
WES	Whole exome sequencing
WGS	Whole genome sequencing
WHO	World health organization
XL	X-linked





# 1

## General introduction and outline of this thesis

- 1.1 Intellectual disability or mental retardation, what is in a name?
- 1.2 Intellectual disability, an issue of major personal and social impact
- 1.3 The importance of a causal diagnosis
- 1.4 Yield of previous cohort studies on aetiology of intellectual disability
- 1.5 Historical hallmarks in genetic diagnostics of intellectual disability
- 1.6 Scope and outline of this thesis

## 1.1 Intellectual disability or mental retardation, what is in a name?

The nomenclature of intellectual disability (ID) has frequently been an issue of debate. The terminology to describe individuals with impaired intellectual functioning has changed many times over the years. These name changes reflect the tendency in the attitude of the society to use more respectful and less offensive terms.<sup>1-7</sup> Intellectual disability and mental retardation are two different descriptions of the same phenomenon. The term "mental retardation" was introduced around 1960.<sup>1, 2</sup> During the last decade the term "intellectual disability" is increasingly being used and actually has generally replaced the term "mental retardation."<sup>3, 4</sup> This transition in nomenclature was initiated by organizations that provide health care and social support to people with ID and increasingly gained ground in other areas, such as public authorities and more recently in (clinical) genetics as well. In 2006 the American Association on Mental Retardation (AAMR) changed her name to the American Association on Intellectual and Developmental Disabilities (AAIDD).<sup>4</sup> Furthermore, in October 2010 President Obama signed "Rosa's law", mandating that the term "mental retardation" should be replaced by "intellectual disability" in the federal statutes ([http://blogs.suntimes.com/sweet/2010/10/obama\\_signs\\_rosas\\_law\\_mental\\_r.html](http://blogs.suntimes.com/sweet/2010/10/obama_signs_rosas_law_mental_r.html)).

One of the voiced arguments to change the name is, that it would be less offensive and a better reflection of how we look at people with ID in their social-ecological context. The term "intellectual disability" would better meet the conception that the condition is not an absolute, fixed state, but is rather the result of an interplay between the cognitive abilities of an individual on the one hand, and his or her environment and personalized support on the other hand.<sup>3-5</sup> Opponents of the name change state that the new name is less accurate, because it does not indicate that it is a developmental problem, and that it causes confusion, because it is not associated with a change in the definition.<sup>6</sup> Most international classification systems, such as the Diagnostic and Statistical Manual of Mental Disorders, Fourth revision (DSM-IV) of the American Psychiatric Association and the International Classification of Diseases, Tenth Revision (ICD-10) of the World Health Organization (WHO) are based on the definition by the AAIDD. This definition comprises three criteria: 1) a significant limitation in *intellectual functioning*, 2) a significant limitation in *adaptive behaviour*, like social and practical skills, and 3) an origin before the age of 18 years.<sup>4</sup> Generally, an intellectual quotient (IQ) score equal to or below 70 is used as an indication for presence of ID, though, as is reflected by the definition, just an IQ level equal to or below 70 is not sufficient to speak of ID.

## 1.2 Intellectual disability, an issue of major personal and social impact

Based on the normal distribution of IQ values in the general population, 2-3% of the individuals have an IQ level equal to or below 70. But, as mentioned above, ID is more than just a limitation in intellectual functioning.

In previous prevalence studies estimations range from 0.2 to 8%. This broad range is caused by differences in study design, study population and definition of ID.<sup>8-11</sup> The estimated prevalence of ID in the Netherlands is 0.7%.<sup>12</sup> The prevalence of severe (IQ<50) ID is lower than the prevalence of mild ID (IQ>50) and is less variable between the different prevalence studies. Most studies report a prevalence of severe ID in the range of 0.3-0.5%.<sup>8-11, 13</sup>

Prevalence studies of ID in the Netherlands are mostly based on registration databases of health care providers, including general practice databases and databases of ID care services.<sup>12, 14</sup> Wullink *et al.* used extrapolation methods to calculate the prevalence of ID in the Netherlands based on the prevalence in Limburg, the Netherlands.<sup>12</sup> The most recent estimate of the prevalence by Ras *et al.*<sup>15</sup> also incorporates insights from international literature and calculated a total prevalence of at least 120.000 individuals (0.75%), including 60.000 (0.38%) people with an IQ<50 and at least 60.000 (0.38%) people with an IQ between 50 and 70. It is expected that the latter category in fact should include many more individuals, but they are more difficult to trace in care service registries.<sup>8-11</sup> Most individuals with ID have a lifelong need of care and it is known that they have significantly more physical and mental comorbidity.<sup>16-23</sup> The costs of health care for people with ID amount to a total of 8.8-10% of the national health care budget.<sup>24</sup> This underlines that ID is not only a frequent disorder, but has a major personal and social impact as well.

## 1.3 The importance of a causal diagnosis

ID is one of the main reasons for referral to a clinical geneticist. Until recently, the cause of ID remained unexplained in at least 50% of the affected individuals. Previous cohort studies showed that in 50-70% of the affected individuals the cause is unknown.<sup>25-30</sup> These percentages concern populations that have been evaluated by clinical geneticists. In practice, the number of people with unexplained ID may be even higher, because many individuals, especially adults, have never received a genetic diagnostic assessment.<sup>31</sup> Likely, many individuals with mild ID have not received such an assessment as well, because a multifactorial cause is suspected. The importance of identification of the underlying cause in an individual with ID is

often undervalued. "What is the benefit for the affected individual him- or herself to know the cause?" is a frequently asked question. However, knowing the cause is of great importance to both affected individuals and their families. It may provide insight in comorbidity, associated behaviour problems, prognosis and lifespan, and recurrence risk, and thereby gives answers to important questions from involved families and health care providers. Identification of a genetic cause precludes further unnecessary and often incriminating testing, and fruitless interventions. Instead, knowing the cause enables specific anticipation on associated health and behavioural problems, since the extreme heterogeneity in aetiology and clinical presentation goes along with diverse prognoses and variable needs. Besides, the emotional impact on affected individuals and/or their families of just getting an explanation why this happened to him/her, to their son, daughter, brother or sister, is enormous in many cases. It is often a great relief when a diagnosis is established and all pieces come together, sometimes after many years of uncertainty and searching for a diagnosis. In addition it enables counselling of recurrence risk, and prenatal testing if indicated and desirable. Moreover, the identification of genetic defects associated with ID provides insight in the underlying pathological mechanisms, which is the first crucial step that opens the way towards the future development of treatment strategies.<sup>31, 32</sup>

## 1.4 Yield of previous cohort studies on aetiology of intellectual disability

Causes of ID can be divided into genetic causes and acquired, environmental causes. In individual cases, the overall phenotype may also be the result of the interplay of both genetic and environmental influences. Most severe forms of ID (IQ<50) have a genetic cause.<sup>11</sup> By contrast, mild forms of ID, especially those with IQ levels around 70, are thought to represent the lower end of the normal IQ distribution and to be more commonly the result of the combined contribution of several genetic factors as well as environmental factors.<sup>11</sup> Genetic causes of ID can be roughly subdivided in chromosomal aberrations, monogenic defects (including autosomal recessive metabolic disorders) and imprinting/epigenetic disorders. A special subgroup of metabolic disorders includes disorders caused by mitochondrial DNA defects. In epigenetic disorders, mutations in a single gene can lead to distortion of the expression of several other genes, without affecting the genomic architecture of these genes. Imprinting is another mechanisms involved in epigenetic disorders and refers to a parent of origin effect on the expression of genes. Chromosomal aberrations can be further subdivided into microscopically visible (numerical or large structural abnormalities) and submicroscopic aberrations.



In previous cohort studies on the aetiology of ID, the percentage of causal diagnoses and the proportion of different diagnostic categories is quite variable. These differences are related to factors as study methodology, characteristics of the study population (age and sex distribution and level of ID), definition and categorization of diagnoses, and availability and application of genetic and other diagnostic tests (such as metabolic screening and neuro-imaging).<sup>9, 25-30</sup> These factors hinder the comparison of the diagnostic yield of previous cohort studies.<sup>29</sup>

**Table 1** gives an overview of previous cohort studies before the era of routine use of genome-wide array analysis in diagnostics of ID. These studies reported a diagnostic yield between approximately 30% and 50%.

In the Netherlands, Van Buggenhout *et al.* (2001)<sup>30</sup> and van Karnebeek *et al.* (2005b)<sup>26</sup> performed systematic aetiologic surveys in Dutch populations of individuals with ID. Van Buggenhout *et al.* examined a cohort of 471, mainly male individuals with an age ranging from 3 to 75 years, living in residential settings of health care providers in the Netherlands. They performed cytogenetic studies, fragile X screening by analysis of CGG repeat expansion in *FMR1*, metabolic tests and in a small subset specific DNA diagnostic tests and brain imaging. They defined a diagnosis in about 48.8%, comprising both genetic (34.2%) and acquired disorders (14.6%). In 1.7% a central nervous system malformation was established as the causal diagnosis. Not all monogenic diagnoses were molecularly confirmed and metabolic disorders (3.4%) were classified as autosomal recessive monogenic disorders. The study of van Karnebeek and others<sup>26</sup> was performed among a group of about 281 male and female children who were referred to a paediatric/clinical genetics department for diagnostic evaluation of unexplained ID between 1998 and 2000. Diagnostic evaluations comprised cytogenetic, molecular and metabolic studies, neurologic examination, and in a minority evaluation by other medical specialists. In 30.6% (86/281) a diagnosis that was classified as "certain" was established, comprising in 15.6% diagnoses confirmed by genetic and/or metabolic tests, in 13.9% clinical diagnoses, and in 1.1% acquired disorders. Compared to the study of van Buggenhout *et al.*, it is remarkable that van Karnebeek *et al.* established a significantly lower percentage of cytogenetic diagnoses. This is explained by the fact that they did not include cases of trisomy 21, which comprise a major part of this diagnostic category. In addition, van Buggenhout *et al.* defined substantially less acquired diagnoses. These differences explain part of the difference in diagnostic yield between these two studies. The important difference in study population, with an overrepresentation of males in the study of van Buggenhout *et al.* likely influences these numbers as well, as is reflected by the higher yield of Fragile X studies in the study of van Buggenhout *et al.*

Stevenson *et al.*<sup>28</sup> reported a diagnostic survey amongst a cohort of almost 11,000 individuals with ID. They report a genetic diagnosis in 28%. However, they included

the diagnostic categories *multifactorial syndromes* and *culturofamilial ID*, which they considered to be partly genetic. If these are not taken into account the total of genetic diagnoses sums up to 21%. Adding 17% acquired diagnoses, the diagnostic yield of this study was 38%. Rauch *et al.*<sup>27</sup> analysed the diagnostic yield of conventional karyotyping, subtelomeric Fluorescent in Situ Hybridization (FISH) screening, molecular karyotyping, and specific DNA diagnostic tests and targeted FISH analyses based on clinical evaluation in a cohort of patients who had mostly an age below six years. In 20 of the total group of 670 patients they also performed molecular karyotyping by 10K array analysis. They report in 39.5% a genetic diagnosis, including in 35.5% a pertinent, molecularly confirmed genetic diagnosis and in the remaining a "clinically unambiguous known syndrome", and/or an unclassified disorder with evidence of Mendelian inheritance by means of pedigree or X-inactivation pattern (4%). Adding 1.3% acquired diagnoses, the total yield of their study amounts to a total of 40.8%.

Other studies reviewed the diagnostic yield of a number of previous studies. In 2005, van Karnebeek *et al.*<sup>25</sup> published a systematic review based on 126 previously published studies on the diagnostic yield of six major diagnostic investigations used in previous cohort studies, including cytogenetic studies, subtelomere FISH studies, cytogenetic and molecular fragile X investigations, metabolic investigations and neuroimaging. They also observed a wide range in diagnostic yield between the several studies. Recently, Michelson *et al.*<sup>33</sup> published a systematic review of the diagnostic yield of genetic and metabolic evaluation in children with global developmental delay or ID. They assessed the yield of G-banded karyotyping (at least 4%), subtelomere FISH (3.5%), microarray analysis (7.8%-10.6%) and metabolic tests (0.2-4.6%). Testing of *FMR1* was positive in at least 2% of patients with mild-moderate ID, and testing of *MECP2* in females with moderate to severe ID had an average yield of 1.5% in the studies they reviewed. These numbers are average numbers, but the results of individual studies showed a much broader range.<sup>33</sup>

Several studies focused on the evaluation of the yield of one or more specific genetic diagnostic tests. Knight *et al.* (1999)<sup>34</sup> showed that subtelomere FISH tests in children with unexplained ID had a high detection yield of subtle chromosomal aberrations of 7%. Using subtelomeric Multiplex Ligand Probe Amplification (MLPA), Koolen *et al.* (2004)<sup>35</sup> identified in a cohort of 210 children with unexplained ID a clinical relevant aberration in 6.3%, 5.1%, and 1.7% of the children with severe, moderate and mild ID respectively.

The majority of the aforementioned studies were performed before the era of the genome-wide array analysis technologies had started. After the introduction of these technologies, several studies on the diagnostic yield of genome-wide array analysis were reported. These studies have been reviewed by Hochstenbach *et al.* (2009)<sup>36</sup>, Sagoo *et al.* (2009)<sup>37</sup> and Miller *et al.* (2010).<sup>38</sup> These studies together

**Table 1** Yield of previous systematic cohort studies on the aetiology of ID before the genome-wide array analysis era

Study	Van Buggenhout <i>et al.</i> , 2001 <sup>30</sup>	Stevenson <i>et al.</i> , 2003 <sup>28</sup>	Van Karnebeek <i>et al.</i> , 2005b <sup>26</sup>	Rauch <i>et al.</i> , 2006 <sup>27</sup>
Patient cohort	471, mainly male patients, age range 3-75y, IQ<50 in >80%	10,997 patients, 59% males, 57% >20 y, IQ<50 "overrepresented"	281 patients, 52% male patients, all children, IQ<50 in 45%	670, mostly with an age<6 y, male:female ratio not mentioned;
Cytogenetic diagnoses	<b>21.2%</b>	<b>11%</b>	<b>11%</b>	<b>30.7%</b>
Conventional karyotyping	21.2%	~11%	7.8%	17.5%
• Trisomy 21	• 18%	• 8.1%	• -	• 9.2%
• Other	• 3%	• 2.9% (in small subset subtelomere anomalies) <sup>c</sup>	• 7.8%	• 8.3%
(Subtelomere) FISH/MLPA	-	- <sup>c</sup>	3.2%	6.6%
(Molecular cytogenetic)	-	-	-	(6.6)
Monogenic diagnoses	<b>13%</b>	<b>8%<sup>d</sup></b>	<b>2.8%</b>	<b>4.8%</b>
Fragile X	3.2%	1.7%	0.4%	1.2%
Other	9.8% <sup>a</sup>	5.3% <sup>d</sup>	2.4%	3.6%
Metabolic diagnoses	- <sup>a</sup>	Not mentioned <sup>e</sup>	1.8% <sup>g</sup>	Not mentioned <sup>l</sup>
Clinical diagnosis, not molecularly confirmed	- <sup>a</sup>	2%	13.9%	4%
Total genetic cause	34.2%	21%	29.5	39.5
Total acquired cause	14.6%	17%	1.1%	1.3%
Total diagnosis (genetic + acquired)	48.8% <sup>b</sup>	38% <sup>f</sup>	30.6% <sup>h</sup>	40.8%

<sup>a</sup> Metabolic diagnoses (3.4%) and clinical diagnoses were included in the group of monogenic diagnoses  
<sup>b</sup> excluding 1.7% diagnosed with a CNS malformation <sup>c</sup> In the paper of Stevenson *et al.*, the percentages of the subgroups numerical, structural and subtelomere anomalies are not further quantified, together they amount to a total of 2.9% <sup>d</sup> It is not indicated whether all cases were molecularly confirmed. Therefore, this number may be lower and the percentage of clinical diagnoses higher. In addition microdeletions associated with Prader-Willi, Angelman, Williams an velocardiofacial syndrome were included in this category <sup>e</sup> Metabolic diagnoses are not mentioned separately, likely these were included in the category monogenic disorders <sup>f</sup> Stevenson *et al.*, also included the diagnostic categories *multifactorial*, *culturofamilial* (together 8%). These were not taken into account here <sup>g</sup> Metabolic studies were performed in a subgroup of 216 patients <sup>h</sup> Van Karnebeek *et al.* divided the diagnostic categories into *certain* and *probable*. Only diagnoses from the category *certain* were taken into account here <sup>i</sup> Rauch *et al.*, performed a pilot with 10K SNP array analysis in 20 patients and extrapolated their results to the total group <sup>j</sup> Rauch

*et al.* included a diagnostic category *mendelian disorders*. It might be that some metabolic disorders were included in this category or in the category monogenic disorders. The category *Mendelian disorders* was not taken into account here, because the diagnoses in this category were not molecularly confirmed.

showed an average diagnostic yield of 15-20%, which compared to conventional cytogenetic tests represents an additive yield of 8-12%.<sup>36-38</sup> Patients suspected of Down syndrome were not included in these results.

1.5 Historical hallmarks in genetic diagnostics of intellectual disability

The yield of cohort studies on the genetic aetiology of ID is apparently highly dependent on the selected patient cohort, the type and resolution of available genetic tests, and the contemporary genetic knowledge. Since the discovery of the first chromosomal disorder in 1959 by Lejeune (trisomy 21), major technological

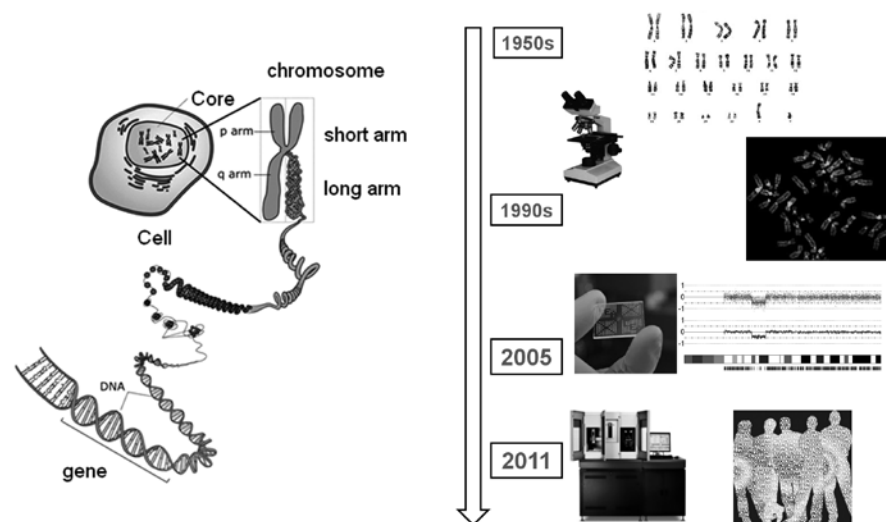
progress in genetic diagnostics has been made.<sup>39</sup> In **table 2** the most important historical hallmarks in genetic diagnostic technologies of ID are represented. (see also figure 1)

Especially during the recent years rapid progress has been made in the development of new genetic technologies that enabled the detection of genetic variants with increasing resolution. In diagnostics of ID, conventional cytogenetic studies have been replaced by genome-wide array analysis in a short period of time.<sup>38, 39</sup> The introduction of genome-wide array technologies has led to the identification of numerous novel microdeletion and microduplication syndromes, which previously escaped detection by routine cytogenetic and molecular cytogenetic techniques.<sup>41-52</sup> The phenotypic characteristics of some microdeletion syndromes are caused by haploinsufficiency of single genes, such as *EHMT1* in Kleefstra syndrome<sup>58</sup>

Table 2 Historical hallmarks in genetic diagnostic technologies of ID

Date	Technique	Detection/resolution	Yield
1950s	Conventional karyotyping	Numerical and gross structural chromosomal disorders. 1959: trisomy 21 identified as the cause of Down syndrome	Together ~4.5-6.5% (excluding trisomy 21) In ~8% trisomy <sup>21, 26-28, 30, 33, 40</sup>
1970s	Banding techniques + cell cycle synchronization	Chromosomal rearrangements (translocations, inversions, deletions-duplications 3-5 Mb)	
1990s	Sanger sequencing	Single nucleotide change	Highly dependent on recognition of clinical phenotype. Rubinstein-Taybi syndrome (OMIM 180894), Tuberous Sclerosis (OMIM 191100) and ATRX syndrome (OMIM 301040) were amongst the first syndromes in which the causative genes were identified. <sup>32</sup>
Mid 1990s	Fluorescent In Situ Hybridization (FISH)	Clinically recognizable microdeletion syndromes Subtelomere microdeletions (later replaced by MLPA)	~6-10% in total <sup>33-35, 40</sup>
Mid 2000s	Genome-wide array analysis	Increasing resolution, up to < 1 kb Small microdeletion and duplication syndromes <sup>41-52</sup> Identification of causal genes in shortest regions of overlap <sup>47, 50, 53-58</sup>	~15-20% (including chromosomal aberrations that can be detected by conventional cytogenetics as well <sup>33, 36-38</sup> )
2009 (research) 2011 (diagnostics)	Next generation sequencing (NGS): <i>Targeted sequencing</i> <i>Whole exome sequencing</i>  Near future: <i>Whole genome sequencing</i>	Single nucleotide change Autosomal dominant <i>de novo</i> , autosomal recessive and X-linked monogenic disorders	Systematic studies evaluating the <i>diagnostic</i> yield of the various NGS approaches are not yet available. Several approaches have been evaluated in research setting: - Whole exome, family based trio sequencing approach (dominant <i>de novo</i> mutations): Vissers <i>et al.</i> Identified in 6/10 individuals a causative mutation <sup>59</sup> - Whole X-exome: Kalscheuer <i>et al.</i> estimated that up to 70% of X-linked ID can be explained <sup>60</sup> - Targeted, 86 known X-linked genes: Hu <i>et al.</i> <sup>61</sup> reported in 42% of X-linked families potentially disease-causing sequence variants - Targeted, homozygous regions (autosomal recessive mutations): Najmabadi <i>et al.</i> <sup>62</sup> reported in 54% of included families potentially disease-causing sequence variants in known and novel candidate ID genes

**Figure 1** Hallmarks in genome-wide diagnostics of intellectual disability



This figure illustrates the major hallmarks in the development of genetic technologies for genome-wide screening in diagnostics of ID.

(previously named 9q subtelomeric deletion syndrome), *RAI1* in Smith-Magenis syndrome<sup>63</sup>, *TCF4* in Pitt-Hopkins syndrome<sup>56</sup> and *KANSL1* in 17q21.31 microdeletion syndrome.<sup>55</sup> Moreover, the application of genome-wide array technologies with increasing density of probes and the study of overlapping microdeletions has led to the identification of several genes associated with developmental disorders or abnormal brain development.<sup>64, 65</sup>

The recent implementation of next generation sequencing (NGS) has marked a revolution in research and diagnostics of ID. Whole-exome sequencing (WES) offers the opportunity to identify disease causing variants throughout the whole human exome. Recently, several studies showed that WES is effective in the identification of genes involved in ID. A family based trio screening approach was successfully applied to identify *de novo* autosomal dominant causes of ID.<sup>59</sup> In addition, WES in clinically recognizable syndromes has led to the identification of

*de novo* gene mutations in an increasing number of ID-associated syndromes, such as Kabuki syndrome<sup>66</sup>, Schinzel-Gidion syndrome<sup>67</sup>, Bohring-Opitz syndrome<sup>68</sup> and KBG syndrome.<sup>69</sup> Furthermore, targeted NGS was successfully used in identification of ID related genes on the X-chromosome and genes involved in autosomal recessive ID in consanguineous families.<sup>61, 62</sup>

Application of WES will increase the proportion of individuals in whom a genetic cause for their ID can be established. The rapid expansion of genes involved in ID by NGS approaches including WES will shed light on the spectrum of genetic causes of ID and corresponding phenotypes. Identification of novel genes and subsequent functional studies will provide new insight in underlying pathogenic mechanisms and involved molecular and biological pathways, which is the first important step towards development of treatment strategies. Moreover, it is expected that in the near future whole-exome sequencing will be followed-up by whole-genome sequencing in the application of gene identifying strategies in ID.<sup>70</sup> This technique will also be able to uncover disease causing variants in non-coding regulatory sequences, such as intronic variants which may lead to alteration of expression and splicing, and may elucidate novel epigenetic causes of ID.

It is obvious that this rapid progress in genetic diagnostic technologies will have great implications and will provide new challenges in care and counselling of individuals with ID and their families.

## 1.6 Scope and outline of this thesis

The preceding reflections on the importance of a diagnosis, the large number of undiagnosed individuals with ID and the great new opportunities in genetic diagnostic technologies provided the motivation for the studies described in this thesis. For these studies we selected a large cohort of 253, mainly adult, individuals with unexplained ID (see **chapter 2**). This cohort is selected from a group of more than 2,000 persons with ID, receiving services from three large service providers in the East of the Netherlands. The 253 individuals represented in our study cohort had not received a present-day genetic diagnostic assessment before and might be considered as representatives of the large group of 50-70% individuals with ID that remained undiagnosed before the era of genome-wide array analysis.

The main objectives of this study were:

- 1) to identify genetic causes of ID
- 2) to study and describe the corresponding phenotypes in detail, including the evolution into adulthood
- 3) to assess the diagnostic yield of the currently available genetic diagnostic repertoire and present-day knowledge

4) to implement this novel insights in the care (both diagnostics and management) and counselling of people with rare genetic causes of ID and their families. To achieve these goals, we have used state of the art genetic diagnostic technologies and a multidisciplinary clinical approach bundling the expertise of the clinical geneticist, other medical specialists as the (paediatric) neurologist, (paediatric) metabolic specialist, and the specialist ID physician.

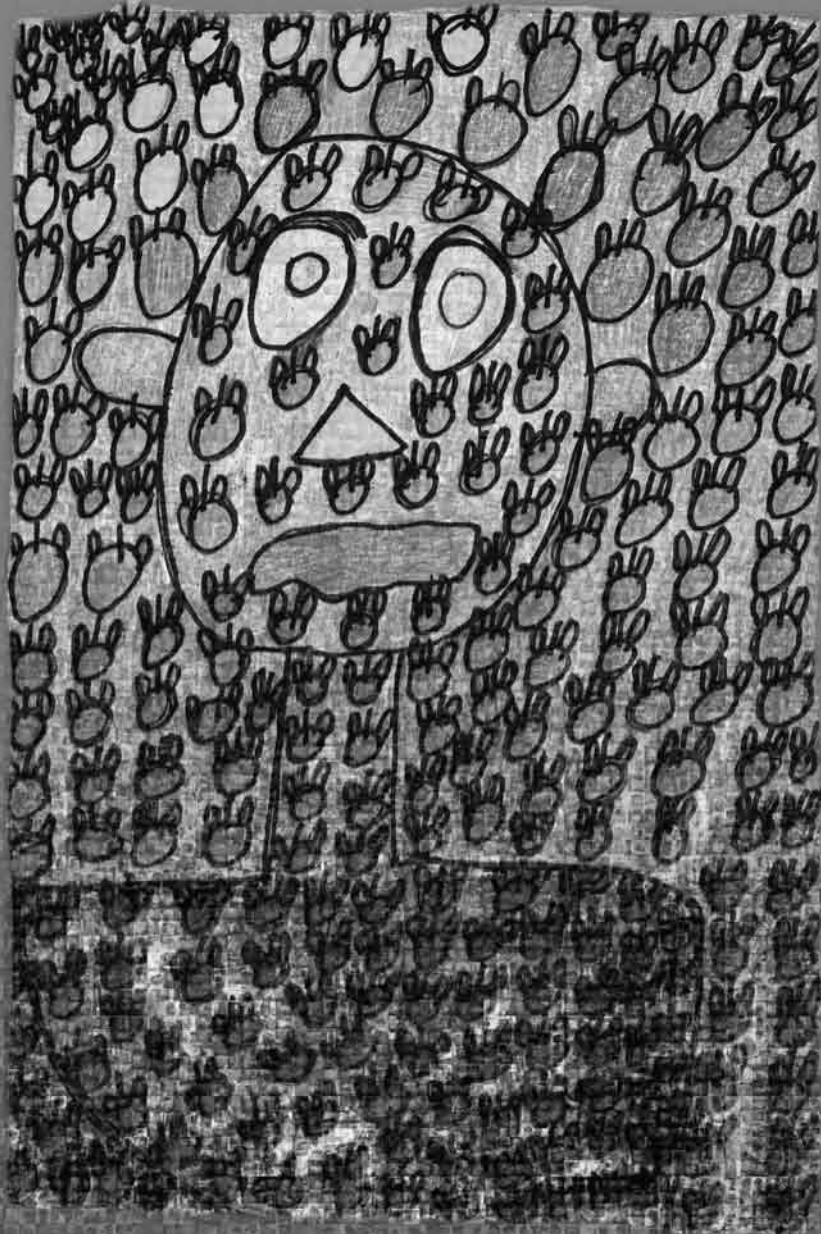
The design of this study, and the selection and recruitment of the patient cohort are detailed in **chapter 2**. **Chapter 3** describes the clinical and molecular characteristics of rarely reported and novel syndromes associated with microscopic (**3.1.1 and 3.1.2**) and submicroscopic (**3.2.1 and 3.2.2**) chromosomal aberrations, and reports the clinical interpretation of X-chromosome copy number variations detected by genome wide array analysis (**3.2.3**). **Chapter 4** includes extensive phenotypic descriptions of established autosomal dominant ID syndromes as Kleefstra syndrome and Angelman- and Rett like syndromes, including the evolution of phenotypes at adulthood. In **chapter 5** the results of application of NGS techniques are presented, leading to a conclusive molecular diagnosis in several ID patients and to the identification and definition of novel genetic disorders with ID. In addition, the identification of several novel candidate ID genes is reported. The overall results of this study are presented in **chapter 6**. The general discussion in **chapter 7** includes reflections on the implementation of the results of this study in clinical practice.

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# 2

## Study design and patient cohort

- 2.1 Organization
- 2.2 Total pre-selection cohort
- 2.3 Patient selection and recruitment
- 2.4 Included subjects
- 2.5 Genetic diagnostic assessment

## 2.1 Organization

This study was embedded in the Dutch research consortium “Sterker op Eigen Benen/Stronger on Your Own Feet”, which is a collaboration between the Radboud University Medical Centre in Nijmegen, The Netherlands, and three service providers for people with intellectual disability (ID) in the eastern part of the Netherlands (*Pluyn*, *Dichterbij* and *Siza*). The consortium facilitates research on behalf of individuals with ID. This specific study was organized within the setting of the department of Human Genetics of the Radboud University Medical Centre. The study was approved by the local ethical committee (NL13636.091.07).

## 2.2 Total pre-selection cohort

Individuals with unexplained ID who participated in this study were selected from a large cohort of 2,069 individuals living in residential settings of *Pluyn*, *Dichterbij* and *Siza*, or receiving ambulatory care of these service providers. The total number of all individuals receiving services of these service providers exceeded this number, but for a large number of individuals insufficient data was available regarding level of ID and/or whether a diagnosis was already known. These individuals were not considered for this study. Available data were derived from the records provided by the care services.

The characteristics of the cohort from which participating individuals were selected (pre-selection cohort) are summarized in **Table 1**. Data regarding age, gender, level of ID and diagnosis are indicated. Diagnostic categories included the categories *unknown diagnosis*, *chromosomal cause*, *monogenic cause*, *metabolic cause*, *clinically recognizable syndrome* and *acquired cause*. Acquired diagnoses for example included perinatal complications and congenital or postnatal infections. For the definition of an acquired diagnosis there had to be sufficient evidence that this diagnosis was the most likely diagnosis. For example, documentation of perinatal asphyxia without confirmation by objective medical data was not accepted as an acquired diagnosis, but was classified as unknown diagnosis. Clinically recognizable syndromes included clinically defined diagnoses without known underlying genetic cause, such as Goldenhar syndrome (OMIM 164210) and Sturge-Weber syndrome (OMIM 185300), or with a known genetic cause, though not molecularly confirmed in the affected individual, such as Borjeson-Forssman-Lehmann syndrome (OMIM 301900), neurofibromatosis (OMIM 162200), Williams syndrome (OMIM 194050) and CHARGE syndrome (OMIM 214800). On average, in 70.9% of the individuals the cause of ID was unknown. This is in agreement with previous studies on aetiology showing that 50-70% of ID is unexplained.<sup>1-6</sup>

Participants of the study were recruited among individuals in the group of 70.9% of individuals with an unknown cause of ID.

Table 1 Overview of the characteristics of the total cohort (pre-selection)	
	Number (%)
Total number of individuals (%)	2069 (100)
Characteristics	
Sex	
Male	1190 (57.5)
Female	770 (42.5)
Age group (y)	
0-18	81 (3.9)
18-40	573 (27.7)
>40	1415 (68.4)
Level of ID	
Mild	461 (22.3/23.7 <sup>d</sup> )
Moderate	711 (34.4/36.5 <sup>d</sup> )
Severe	777 (37.6/39.9 <sup>d</sup> )
Unknown <sup>a</sup>	120 (5.8)
Diagnosis	
Unknown	1467 (70.9)
Chromosomal	331 (16)
Monogenic	31 (1.5)
Metabolic <sup>b</sup>	32 (1.5)
Clinical Syndrome <sup>c</sup>	68 (3.3)
Acquired	140 (7.1)

<sup>a</sup> The exact level of ID was not documented, but the IQ level was at least  $\leq 70$ .  
<sup>b</sup> Metabolic causes often have an autosomal recessive inheritance, but in almost all individuals with a metabolic diagnosis, the diagnosis was based on metabolic examinations and not confirmed by molecular genetic tests. Therefore, these were classified in the metabolic category instead of the monogenic category.  
<sup>c</sup> Though the underlying genetic cause of several clinical syndromes has been elucidated, this was not yet molecularly confirmed in affected individuals assigned to this diagnostic category.  
<sup>d</sup> calculated % if people with unknown level of ID are not taken into account.

2.3 Patient selection and recruitment

Selection of study participants from the total pre-selection cohort was based on the presence of *unexplained* ID (unknown diagnosis group) in combination with presence of one or more additional features as summarized in **Table 2**. The second criterion was used to increase the likelihood that a genetic diagnosis would be

found, since it was assumed that the likelihood of a genetic cause is higher in familial ID and in ID in combination with additional features.<sup>7,8</sup>

Table 2 Selection criteria	
1.	<b>Presence of <i>unexplained</i> ID</b> (IQ $\leq$ 70 or a developmental age equivalent of $\leq$ 12 years)
2.	<b>Presence of <math>\geq</math>1 of the features below (HPO* terms)</b> Short stature or overgrowth (height $\leq$ -2 SD or $\geq$ +2 SD) Microcephaly or macrocephaly (head circumference $\leq$ -2 SD or $\geq$ +2 SD) Facial dysmorphism Congenital abnormality (non-CNS) (i.e. cardiac, renal, skeletal, gastro-intestinal, urogenital, craniofacial, skin/hair/nail anomalies) Abnormality of the CNS (i.e. disorders of the central and /or peripheral nervous system, extrapyramidal and /or cerebellar disorders, regression and/or neurodegenerative signs) Familial ID or consanguinity

\*HPO: Human Phenotype Ontology (<http://www.human-phenotype-ontology.org/>)

Further selection of candidate participants was based on the data in the medical files of the service providers. Only individuals of whom sufficient data were available to determine whether they fulfilled the criteria for selection were taken into account. The parents or legal representatives of selected individuals were asked for their informed consent by a letter including information about participation in the study and an informed consent form. They had the possibility to indicate either permission or decline of participation. Some parents/legal representatives mentioned their reasons for decline. In a minority of the cases the parents/legal representatives were personally approached by a specialist ID physician of one of the participating care services. In total, the parents or legal representatives of 838 individuals were approached. Informed consent was obtained for 270 individuals (32.2%). Informed consent was declined for 280 individuals (33.4%). The parents or legal representatives of 288 individuals (34.4%) did not reply (see **Figure 1**). Hundred nineteen of 280 (42.5%) parents and/or legal representatives who declined participation, mentioned their motives. Most frequent reasons were:

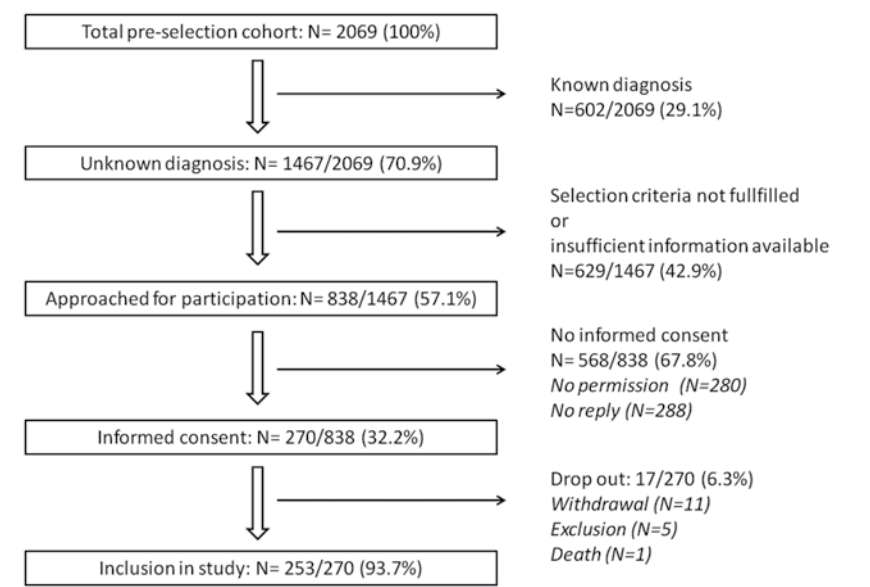
- 1) participation is too incriminating to the candidate participant (in 37%)
- 2) the causal diagnosis is already known according to the parents/legal representative (in 17.6%)
- 3) medical condition and/or behavioural problems of the candidate participant hinder participation (in 13.4%)
- 4) practical reasons, i.e. transport problems, no guidance, no time (in 9.2%)



In the remaining 22.8% various other reasons were mentioned, including: genetic investigations have already been performed, the participant does not benefit from the investigations, the investigations have no use.

Seventeen of 270 participants dropped out after initial inclusion. One individual died and in eleven cases the parents or legal representatives decided to withdraw their permission, for example because of practical reasons. In one case, it turned out that a genetic diagnosis had already been established. Four individuals were excluded because, after initial inclusion, further evaluation turned out that they had an IQ level above 70 and thus did not fulfill the selection criteria (see **Figure 1**). In total 253 of the total cohort of 2,069 individuals (12.2%) were included in this study. After obtaining written permission from the parents or legal representative for participation, patients were invited to visit the outpatient clinic of the department of Human Genetics of the Radboud University Medical Centre in Nijmegen for an extensive multidisciplinary clinical evaluation (see paragraph **2.5**).

**Figure 1** Flowchart of patient selection and recruitment



## 2.4 Included subjects

In total 253 individuals from 234 families participated in the study. Detailed characteristics regarding gender and age distribution, distribution of the level of ID, type and number of selection criteria and familial cases are represented in **Table 3**. The majority of the participants had an adult age above 40 years. Male to female ratio was 1.75:1, which corresponds with the overrepresentation of males in the total population of people with ID.<sup>9</sup>

## 2.5 Genetic diagnostic assessment

The study was divided into two phases:

- 1) the diagnostic phase and
- 2) the diagnostic related research phase

During the first, diagnostic phase we performed clinical investigations and used genetic diagnostic tests that are currently routinely used in clinical genetic practice.

### Clinical investigations

In collaboration with the department of primary care of the Radboud University Medical Centre, chair “health care for people with ID”, we have established a multidisciplinary outpatient clinic for the clinical evaluation of the participating patients. At this outpatient clinic, patients were simultaneously seen by a research physician, a clinical geneticist and a specialist ID physician. The evaluation included a thorough medical history taking and physical and dysmorphological examinations. If indicated, other medical specialists, such as a (paediatric) neurologist, (paediatric) specialist metabolic diseases, and a psychiatrist were consulted. During the visit, blood samples were taken from the patients and, if available, from their parents as well. DNA was obtained from peripheral blood cells and extracted according to standardized procedures.

### Molecular genetic analyses in the diagnostic phase (phase 1)

During the first, diagnostic phase, specific DNA diagnostic tests were requested based on the clinical suspicion of a recognizable syndrome in a subset of the patients. Genome-wide microarray analysis to detect (sub)microscopic chromosomal aberrations/copy number variations (CNVs), and a metabolic screen were performed as standard. In addition, DNA samples of patients and available parents were stored for possible future use in both diagnostics and research.

In all patients genome wide SNP (Single Nucleotide Polymorphism) array analysis



Table 3 Characteristics of participants	
Total Number of Index <sup>a</sup> Participants (%)	234 (100)
Patient Characteristics	N (%)
Gender	
Male	149 (64)
Female	85 (36)
Age Group (y)	
0-18	40 (17.1)
18-40	84 (35.9)
>40	110 (487)
Level of ID	
Mild (IQ 50-70)	60 (25.64)
Moderate (IQ 35-50)	60 (25.64)
Severe (IQ<35)	114 (48.70)
Selection Criteria (HPO terms) <sup>b</sup>	
Short stature or overgrowth <sup>c</sup>	70 (29.9)
Microcephaly or macrocephaly <sup>d</sup>	64 (27.4)
Facial dysmorphism	119 (50.9)
Congenital abnormality (non-CNS)	74 (31.6)
Abnormality of the CNS	63 (26.9)
Familial ID or consanguinity	99 (42.3)
(Epilepsy) <sup>e</sup>	93 (39.7)
Number of Positive Selection Criteria	
1	84 (35.9)
2-3	121 (51.7)
4-5	27 (11.5)
>5	2 (0.9)
Familial Cases	
Sibpair	13 (+13)
>2 siblings	2 (+5)
Mother-child	1 (+1)
Total Number of Individuals <sup>f</sup>	253 (234+19)

CNS= central nervous system  
<sup>a</sup> excluding participating affected family members (see familial cases)  
<sup>b</sup> several participants fulfilled more than 1 selection feature  
<sup>c</sup> short stature: height  $\leq$  -2 SD; overgrowth height  $\geq$  +2 SD  
<sup>d</sup> microcephaly: head circumference  $<$ -2 SD/ $<$ P2; macrocephaly: head circumference  $>$ +2 SD/ $>$ P98  
<sup>e</sup> epilepsy was not used as a selection criterion  
<sup>f</sup> including participating affected family members

was performed according to the standard Affymetrix GeneChip protocol (Affymetrix, Inc., Santa Clara, CA, USA). In the majority of the individuals the Affymetrix 250k Nspl SNP array platform was used. More recently, the Affymetrix 2.7M array platform was used, due to its introduction as the standard array platform in the diagnostic services at our department later during the study. Copy number estimates were determined using the updated version 2.0 of the CNAG (Copy Number Analyzer for Affymetrix GeneChip mapping) software package (Affymetrix 250k SNP array) or the ChAS (Chromosome Analysis Suite for Affymetrix GeneChip mapping) software package (Affymetrix 2.7M array). The normalized ratios were subsequently analyzed for genomic imbalances by a standard Hidden Markov Model (HMM). Copy number variations (CNVs) were mapped according to the UCSC genome browser build May 2004 (<http://genome.ucsc.edu/>; NCBI35/Hg17) for the Affymetrix 250k array platform and to build March 2006 (NCBI36/Hg18) for the Affymetrix 2.7M array platform. Subsequently, Mb positions of CNV breakpoints were converted to the latest version of the UCSC genome browser (Hg19 assembly, February 2009). The Affymetrix 250k SNP array platform in addition allowed the detection of homozygous regions on the chromosomes, which are possible candidate regions for the localization of genes involved in autosomal recessive (AR) forms of ID. In all patients a standard metabolic screen in serum and, if available urine, was performed. This included measurement of amino acids, carnitine and acylcarnitines, quantitative creatine biosynthesis, very long chain fatty acids, sialotransferrines and lactate in serum; and amino acids, organic acids, uric acid, creatinine, purines and pyrimidines, and mucopolysaccharides and oligosaccharides in urine. On clinical indication screening tests for lysosomal disorders were included as well. In all patients, except for those with microcephaly, DNA analysis of the *FMR1* gene was performed to exclude Fragile X syndrome. When possible, segregation of genetic/chromosomal variants was tested in the parents and/or other family members by SNP array, Multiplex Ligation-dependent Probe Amplification (MLPA), Fluorescence In Situ Hybridization (FISH) or DNA analysis of the specific gene mutation.

**Molecular genetic analysis in the diagnostic related research phase (phase 2)**  
About one quarter (24.6%) of the patients in whom a genetic diagnosis could not be established in the first phase of the study, were further analyzed by various next generation sequencing (NGS) approaches. These patients could be roughly divided into two groups 1) patients with sporadic ID and 2) patients with familial ID, further subdivided into patients from families with an X-linked (XL) pedigree and patients from families with an AR pedigree, with or without the presence of homozygous regions  $\geq$ 3 Mb.

Isolated patients were considered for the involvement of *de novo* mutations with a dominant effect. Hence, these patients were potential candidates for family-based whole-exome sequencing (WES). Patients with an IQ<50, normal karyotype, negative family history and availability of DNA samples from both of the parents were selected for this approach, as previously described by Vissers *et al.*<sup>10</sup> and described in **chapter 5.2** of this thesis.<sup>11</sup> The analysis also allows the identification of recessive mutations, though these patients were selected *against* AR inheritance patterns.

Index patients with familial ID were included in further NGS studies including WES or X-exome sequencing. In three AR families, homozygosity mapping as described by Schuurs-Hoeijmakers *et al.*,<sup>12</sup> was performed initially, followed by direct Sanger sequencing of candidate genes in the overlapping homozygous regions. In index patients from families with a pedigree that could both fit an AR and XL inheritance pattern, WES was performed to search for causative homozygous, compound heterozygous and X-linked mutations. Index patients from families compatible with XL inheritance were candidates for X-exome sequencing in collaboration with the Max Planck Institute for Molecular Genetics in Berlin.<sup>13</sup>

Validation and segregation analysis of putative causative variants were done by Sanger sequencing.

### Assessment of clinical relevance

Abnormal findings in both the diagnostic and diagnostic related research phase of the study, including chromosomal CNVs, intragenic variants and abnormalities in metabolic tests, were classified into three different categories reflecting the presumed clinical relevance. The classification categories include:

- 1) (likely) pathogenic: in case of a pertinent or plausible causal relationship with the observed phenotype
- 2) unknown clinical relevance: in case of limited or absent evidence for a direct causal relation. However, the clinical relevance of the respective variant cannot be excluded.
- 3) (likely) non-pathogenic: in case a relation with the observed phenotype was pertinent absent or very unlikely

**Table 4** shows a schematic overview of the general criteria that were considered to assess clinical relevance and to classify abnormal findings into these three categories. Classifications of findings in each individual patient in this cohort were based on careful consideration of the full picture of the criteria for assessment of clinical relevance as mentioned in Table 4. None of the criteria was absolute and each criterion was considered in perspective to the other criteria, the currently available information in literature and databases, and the phenotype of the patient.

Microdeletions and microduplications that can be considered as a strong risk factor for ID but have sporadically been reported in healthy controls or are inherited, such as 16p11.2 microdeletions<sup>14</sup> and 15q13 microdeletions and duplications<sup>15</sup>, were either classified as (likely) pathogenic or as of unknown clinical significance depending on the phenotype of the respective patient. For example, if a CNV was detected in a patient with a very severe phenotype that did not resemble the phenotype in other patients with a similar CNV, it was assumed that other yet undetermined factors have contributed to the phenotype. In these cases, the particular CNV was classified as "unknown clinical relevance", whereas in a patient with a mild phenotype that resembles the phenotype in other patients with a comparable CNV, the same CNV could have been classified as "(likely) pathogenic".

**Table 4** Criteria for assessment of clinical relevance, grouped by the three different classification categories

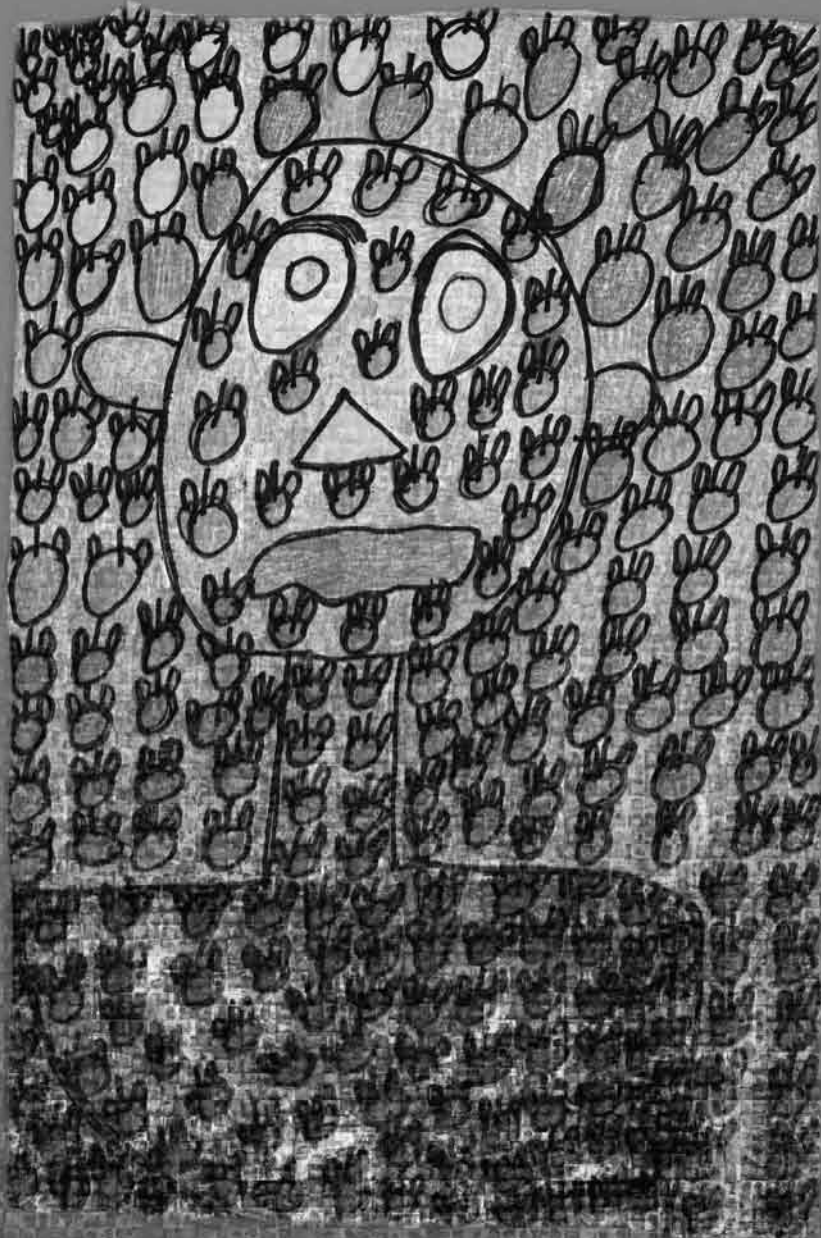
Criteria	Classification		
	<i>(Likely) pathogenic</i>	<i>Unknown</i>	<i>(Likely) non-pathogenic</i>
Inheritance	De novo occurrence or segregation with the phenotype in familial ID	Segregating with the phenotype, but other variants present as well	Presence in a non-affected family member or not segregating with the phenotype
Occurrence in patients and controls (databases: DGV, dbSNP, ECARUCA, DECIPHER, in house databases)	Similar genetic defects in patient(s) with overlapping phenotype No reports in healthy controls	Low detection rate in apparently healthy controls, but higher frequency in individuals with ID	Polymorphism in healthy controls No segregation with the phenotype within the family
Size and gene content (CNVs)	Size (>1.5 Mb) and gene content (>20 genes)	Small number of genes that have not been previously related to ID	No genes involved
Known association with ID of involved genes	CNV overlaps with known ID gene Mutation in known ID gene	Role of involved gene(s) is not known	Gene involved is not associated with ID phenotype
Function and expression of involved genes	Role in pathway involved in ID and/or brain development/functioning Brain specific expression of involved gene(s)	Unknown function and/or unknown or ubiquitous expression pattern of involved gene(s)	Involved gene does not play a role in pathway involved in ID or brain development/functioning Gene is not expressed in brain
Type of mutation, Conservation, biochemical difference, prediction programmes	Nonsense, frameshift Highly conserved missense (PhyloP>3), high Grantham score (>50) Predicted to be deleterious by SIFT and POLYPHEN	Missense Moderately conserved (PhyloP 1-3) Moderate Grantham score (25-50) Predicted to be deleterious by one of the prediction programs and tolerated by the other	Synonymous Poorly conserved (PhyloP<1) Low Grantham score (<25) Predicted to be tolerated by SIFT and POLYPHEN
Metabolic disorder	Repeatedly abnormal values of metabolites in serum and/or urine that could fit with the phenotype and are not explained by medication or nutrition (i.e. anti-epileptic drugs)	Consistent aspecific abnormal values of metabolites in serum and/or urine with unknown association with the phenotype	Abnormal values of metabolites in serum and/or urine that have no association with the ID phenotype and/or have an iatrogenic source.
Mouse models	Central nervous system or (neuro)developmental disorder and/or lethal phenotype	Unknown phenotype	No phenotype

DGV: database of genomic variants (<http://projects.tcag.ca/variation/>); dbSNP: the single nucleotide polymorphism databases (<http://www.ncbi.nlm.nih.gov/projects/SNP/>); ECARUCA: European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations (<http://umcecaruca01.extern.umcn.nl:8080/ecaruca/ecaruca.jsp>); DECIPHER: Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources (<http://decipher.sanger.ac.uk/>)

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# 3

## Identification and characterization of chromosomal disorders with ID

### 3.1 Microscopic chromosomal aberrations

- 3.1.1 Further Molecular and Clinical Delineation of the Wisconsin Syndrome Phenotype Associated With Interstitial 3q24q25 Deletions  
*Am J Med Genet* 2010;155A:106-112
- 3.1.2 Clinical and molecular characterization of two patients with a 6.75 Mb deletion in 8p12p21 with two candidate loci for congenital heart defects  
*Eur J Med Genet* 2009;52:134-139

### 3.2 Submicroscopic chromosomal aberrations

- 3.2.1 Identification of *ANKRD11* and *ZNF778* as candidate genes for autism and variable cognitive impairment in the novel 16q24.3 microdeletion syndrome  
*Eur J Hum Genet* 2010;18:429-435
- 3.2.2 Chromosome 1p21.3 microdeletions comprising *DPYD* and *MIR137* are associated with intellectual disability  
*J Med Genet* 2011;48:810-818
- 3.2.3 Interpretation of clinical relevance of X-chromosome copy number variations identified in a large cohort of individuals with cognitive disorders and/or congenital anomalies  
*Eur J Med Genet* 2012;Jul 14



# 3.1.1

## **Further Molecular and Clinical Delineation of the Wisconsin Syndrome Phenotype associated with Interstitial 3q24q25 Deletions**

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*Am J Med Genet* 2010;155A:106-112



## Abstract

Deletions of the distal 3q22.3 region encompassing the gene *forkhead transcription factor FOXL2* (*FOXL2*) usually result in intellectual disability (ID) and the highly recognizable blepharophimosis-ptosis-epicanthus inversus syndrome (BPES). We encountered three patients with molecularly defined interstitial deletions distal to the *FOXL2* gene. They present with remarkably similar manifestations comprising variable ID, a coarse facial appearance, including prominent nose and eyebrows, hypogonadism and skin pigmentation abnormalities, and they share an approximately 8.8 Mb overlapping 3q24q25 deletion. Interestingly, one of the present patients was described previously in a clinical report with emphasis on her clinical similarity to the Wisconsin syndrome, suggesting that Wisconsin syndrome might be caused by a (micro)deletion within the 3q24q25 region.

## Introduction

Interstitial chromosomal aberrations of the distal long arm of chromosome 3 are quite rare.<sup>1-2</sup> Most patients with a deletion in this region have blepharophimosis-ptosis-epicanthus inversus syndrome (BPES; OMIM#110100) due to deletion of the gene *forkhead transcription factor FOXL2* (*FOXL2*; OMIM#605597) located in 3q22.3.<sup>3-5</sup> Patients with interstitial deletions of the long arm of chromosome 3 also have intellectual disability (ID), microcephaly, pre-and postnatal growth retardation, congenital heart defects and various skeletal anomalies.<sup>1, 6-19</sup>

In 2003 a female patient with an interstitial deletion in chromosome region 3q23q25 was reported as the second case of Wisconsin syndrome<sup>2, 20</sup> after the first delineation of this syndrome by John M. Opitz in 1976, who described a combination of cranio-synostosis, ID, upslanted palpebral fissures, small ears and short fourth metatarsals with recessed fourth toes as Wisconsin syndrome.

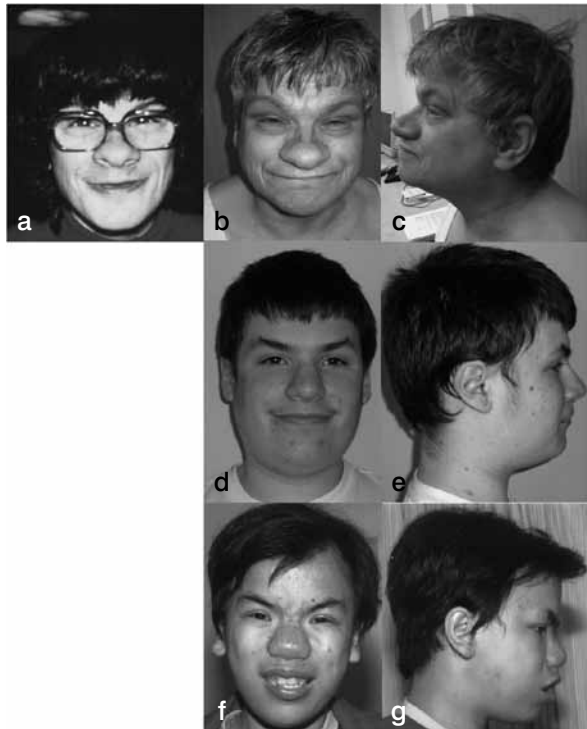
Here we describe the clinical and molecular characteristics of 3 patients resembling Wisconsin syndrome with overlapping interstitial deletions in 3q24q25 that are located distally to the *FOXL2* gene. We compared the clinical and molecular characteristics of these 3 patients with 16 previously published patients with an interstitial deletion overlapping the deletion of one or more of the present patients, both with and without involvement of the BPES region.

## Patients and Methods

### Patient 1

This patient was referred at the age of 60 years. No genetic evaluation had been performed prior to referral. She was the second of 13 children, born to non-consanguineous parents. There was no information available regarding pregnancy and birth. Psychomotor development was moderately to severely delayed. Formal developmental assessment at adult age had demonstrated a developmental level of a 3-year-old child. She had primary amenorrhea, hypermetropia and a bilateral mixed hearing loss of 60 decibels. Behaviour problems included self mutilation and sometimes aggressive outbursts. She had a high pain threshold. At the age of 59 years she received a total hip prosthesis because of arthrosis. At the age of 60 she had a masculine appearance. Her height was 152 cm (<3<sup>rd</sup> centile), weight 66 kg (75-90<sup>th</sup> centile) and head circumference 51 cm (<3<sup>rd</sup> centile). The skull was brachycephalic and she had coarse face with a broad nose with prominent alae nasi and nasal tip, arched eyebrows, midface hypoplasia, macrostomia with full everted lower lip, short philtrum, large protruding tongue and large ears. The neck was broad and short (Fig. 1 A-C). There was no breast development. Axillary and

**Figure 1** Facial appearance of patient 1 at 60 years (b-c) and at younger adult age (a), of patient 2 at age 13 years (d-e) and of patient 3 at age 18 years (f-g) Note the coarse face, prominent nose, full lower lip, large mouth and unusually shaped, bushy eye brows.



pubic hair-growth was normal. External genitalia appeared normal. She showed a mild clinodactyly of the 5<sup>th</sup> fingers and bilateral hockey stick-lines, and flat feet, hallux valgus and normal other toes. She had local hirsutism on the cheeks, toes and ears. The skin at the extensor sides of the wrists showed several hypopigmentation spots. Routine cytogenetic analysis by G-banding showed an interstitial deletion encompassing chromosome bands 3q23 to 3q25.3. Evaluation of the hormonal profile documented hypogonadotropic hypogonadism (LH 0.15 E/l, FSH 0.27 E/l, estradiol 69 pmol/l). Bone densitometry showed signs of osteoporosis, mainly in the lumbar spine. To prevent osteoporotic complications she is now on treatment with bisphosphonates and vitamin D.

### Patient 2

This boy was referred for chromosome analysis at the age of 6 11/12 years because of developmental delay with moderate learning difficulties. He was conceived through assisted conception with Pergonal treatment. Birth was complicated by an emergency caesarian section at 40+6 weeks gestation due to fetal distress. Birth weight was 3,200 g (just below 25<sup>th</sup> centile). Head circumference at birth was 34.5 cm (50<sup>th</sup> centile). The patient was born in good condition but was slow to feed. He was ascertained due to concerns about his development. At 6 months of age he was a quiet baby, with absence of typical babble. At 2 years he was not making any attempts at speech although he had some understanding. He had speech therapy and was initially diagnosed with isolated speech delay and verbal dyspraxia. Recent assessment of his speech at the age of 12 years showed a language level of 6 9/12 years. There were no concerns regarding his early motor development, though he walked at age 2 years. He learned to ride a bicycle at 7 years and has reasonably fine motor skills. There have been no concerns about his hearing but he was diagnosed with myopia requiring glasses at age 12 years. He had concentration problems and preferred to be in a routine, but was generally a co-operative child with good behaviour. He was able to attend mainstream school for his primary education but thereafter attended a special school for children with learning disabilities. He was able to read and write simple sentences. The patient's general health had been good without any hospital admissions. He progressed normally through puberty during his teenage years. On physical examination at the age of 13 years had a head circumference of 57.9 cm (91<sup>st</sup> centile) and a broad nose and a large mouth. The eye-brows were unusually shaped and upsweeping (Fig.1 D-E). The skin was soft with a small hypopigmented patch on the right knee. He had tapering fingers with very mobile joints in his hands. His large joints were hyperextensible and he had a Beighton score of 9. There were no joint contractures. He had some very small areas of alopecia within his scalp hair that had been present since birth. He had bilateral supernumerary nipples. Genitalia were normal with no signs of hypogonadism. Cardiovascular status was normal. Conventional high resolution cytogenetic analysis at the age of 7 years demonstrated a deletion of chromosome bands 3q25.1 to 3q25.33.

### Patient 3

This patient was described previously by Ko et al.<sup>2</sup> Summarized, the phenotype included moderate ID, epilepsy, primary hypogonadotropic hypogonadism, short recessed fourth toes and facial dysmorphism (Fig.1 F-G). Conventional high resolution cytogenetic analysis showed a deletion of chromosome bands 3q23-q25.

### Genome-Wide Array Analysis

To further characterize the cytogenetically detected deletions, we performed genome-



wide array analysis. In patient 1 and 3 genome-wide single-nucleotide polymorphism (SNP) array analysis was performed on DNA with the Affymetrix 250k SNP array platform according to the standard Affymetrix GeneChip protocol (Affymetrix, Inc., Santa Clara, CA, USA). In patient 2 an oligonucleotide array comparative genomic hybridisation (oaCGH) was carried out with patient and normal pooled sex matched control DNA using a customised Agilent 60-mer oligonucleotide array in a 4 x 44K format (NGRL (Wessex) Constitutional Array CGH v2). All deletions were mapped according to the March 2006 assembly of the UCSC genome browser (NCBI Build 36.1/hg18; <http://genome.ucsc.edu>).

## Results

### Clinical manifestations

Clinical findings of the three patients are shown in Table 1 and compared with those observed in 16 patients with an overlapping deletion previously reported and cited in online databases, including the DatabasE of Chromosomal Imbalances and Phenotypes using Ensembl Resources (Decipher) and the European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations (ECARUCA) (Table 1 and Fig. 2). Corresponding traits in these 19 patients are ID (19/19), various skeletal (digital and joint) anomalies (14/19), external ear anomalies (13/19), microcephaly (10/19), pre- and postnatal growth retardation (respectively in 6/19 and 11/19), cardiac anomalies (8/19), ocular problems (8/19) and genital anomalies (4/19 (3/4 males)). The digital anomalies include arachnodactyly, camptodactyly, clinodactyly, syndactyly and brachydactyly. Reported joint anomalies include club feet, hip dysplasia, hyperextensibility and limited/restricted movement or contractures. Cardiac anomalies in 4/8 of the patients were a ventricular septal defect. Reported ocular problems are mostly non specific and variable, including refraction errors and strabismus. More severe reported ocular problems are optic atrophy (in patients 12 and 17) and microphthalmia (patient 9 and 13). Genital anomalies include hypospadias, cryptorchidism, and micropenis.

### Genome-Wide Array Analysis

Further characterization of the deletion in patient 1 by genome wide 250k SNP array analysis (Affymetrix inc, Santa Clara California) showed a 16.2 Mb loss in 3q24q25.33 (Figs. 2 and 3). The final karyotype was formulated as follows: 46,XX,del(3)(q24q25.33).arr snp 3q24q25.33(144,654,575-160,842,543)x1 with flanking normal probes SNP\_A-4209748 and SNP\_A-2244366 at, respectively, 144,621,072 and 160,873,241 Mb on chromosome 3. At present, there are at least 70 known genes in this deleted region of 3q24q25.33. Unfortunately, the patient's parents were not

available for segregation analysis. Further delineation of the deletion in patient 2 by array CGH showed a minimal deletion of 8.83 Mb and a maximum of 8.95 Mb. The proximal breakpoint was within the 121 kb between oligos A\_14\_P106909 at Mb position 150,951,254 and A\_14\_P121635 at Mb position 151,072,501 and the distal breakpoint was within the ~8 kb between oligos A\_14\_P133326 at 159,897,607 and A\_14\_P123284 at 159,905,167. Maternal chromosomes were normal but the paternal chromosomes have not been examined. The final karyotype was formulated as follows: 46,XY,del(3)(q25.1q25.33).arr cgh 3q25.1q25.32(151,072,501-159,897,607)x1. The minimal deleted region contains 42 annotated genes. Genetic and clinical details of patients 1 and 2 have been submitted to the ECARUCA database ([www.ecaruca.net](http://www.ecaruca.net), case ids. 4312 and 4513 respectively). Genome wide 250k SNP array analysis in patient 3 demonstrated a 19.38-Mb loss in 3q24q26.1 (147,27-166,66 Mb (Figs. 2 and 3)). The final karyotype was formulated as follows: 46,XX,del(3)(q24q26.1).arr snp 3q24q26.1(147,273,493-166,655,467)x1, with flanking normal probes SNP\_A-1863440 and SNP\_A-1929743 at respectively 147,267,706 and 166,726,888 Mb of chromosome 3. This region comprises more than 50 genes.

## Discussion

We report on three patients with syndromic ID and an overlapping interstitial deletion in 3q24-q25. The manifestations of these patients are compared with those observed in 16 patients with a previously reported overlapping deletion (Table 1, Fig. 2). In most previously reported patients, except for patient 9,10 and 18, the deletions were identified by conventional cytogenetic studies and thus breakpoint delineations were not as precise as in patients 1, 2 and 3. This hampers exact genotypic comparison between the present and previously reported patients. Genome-wide array analysis in patients 1, 2, and 3 showed that the deletions in these patients did not comprise the 3q23 region, as initially was assumed by conventional cytogenetic examination in patients 1 and 3. The 5 previously reported patients (patients 4-8) with a deletion encompassing the distal boundary of the 3q22.3 region where the *FOXL2* gene is located, showed the highly recognizable BPES phenotype. Five other patients (patients 10-14) who were reported to have a deletion with a proximal delineation at chromosomal band 3q23 detected by conventional cytogenetics, also had BPES. So, most likely these deletions extend beyond the boundary between chromosomal bands 3q22.3 and 3q23. Remarkably, in patient 9 array CGH showed that the *FOXL2* gene was not deleted, though this patient had the classical phenotype of BPES. It was suggested that this might be the result of disruption of regulatory elements regulating *FOXL2* expression [Rea et al., 2010]. Patients 15-19 are the only five previously reported patients with an

**Table 1** Findings in the Patients in the Present Study and 16 Previously Reported Patients With an Overlapping Deletion of the Long Arm of Chromosome 3

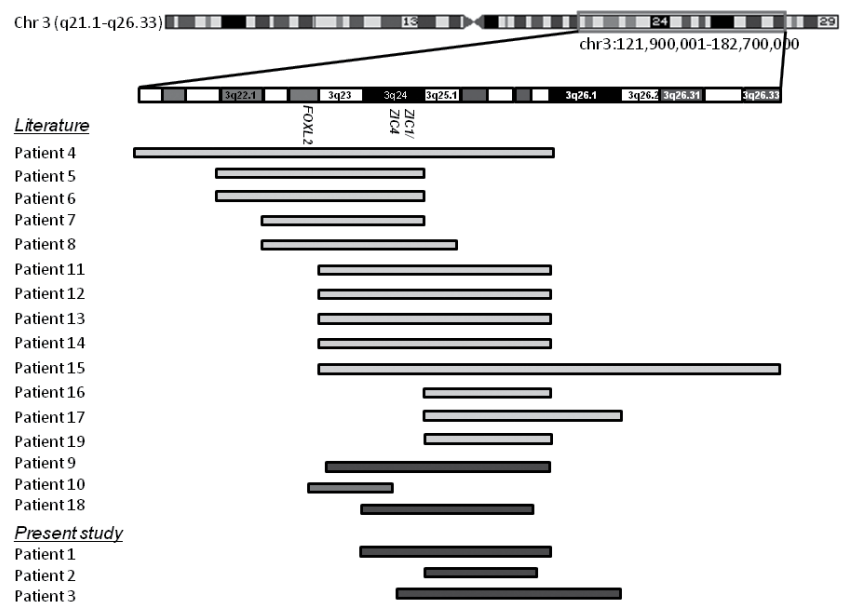
Clinical findings	Present study			Literature					
	Patient 1	Patient 2	Patient 3	4 <sup>1</sup>	5 <sup>13</sup>	6 <sup>5</sup>	7 <sup>16</sup>	8 <sup>16</sup>	9 <sup>19</sup>
<b>Gender</b>	Female	male	female	female	male	male	male	male	female
<b>Deletion 3q</b>	q24q25.33*	q25.1q25.33*	q24q26.1*	q21q25 (mosaic)	q22q24	q22.1q24	q22.2q24	q22.2q25.1	q22.3q25.1*
<b>Biometry</b>	NR	-	-	-	+	+	-	+	-
IUGR	+	-	-	-	+	+	-	+	+
Postnatal GR	+	-	-	+	+	+	-	+	+
Microcephaly	+	-	-	+	+	+	-	+	+
<b>Intellectual disability</b>	moderate-severe	mild	moderate	mild	moderate	severe	mild (speech)	+	+
<b>Facial dysmorphism</b>	-	-	-	+	+	+	+	+	+
BPES	+	-	-	-	-	-	-	-	-
Upslanted palpebral fissures	+	+	+	+	+	+	+	+	+
Unusually shaped eyebrows	+	+	+	+	+	+	+	+	+
Coarse facies	+	+	+	+	+	+	+	+	+
Prominent nose	+	+	+	+	+	+	+	+	+
Ear anomalies	+	+	+	+	+	+	+	+	+
Macrostomia	+	+	+	+	+	+	+	+	+
Full lips	+	+	+	+	+	+	+	+	+
<b>Hypogonadism</b>	+	-	+	NR	NR	NR	NR	NR	NR
<b>Genital anomalies</b>	-	-	hypoplastic uterus	NR	-	cryptorchidism	-	hypospadias	NR
<b>Skeletal anomalies</b>	-	+	+	NR	NR	NR	NR	NR	NR
Digit anomalies	-	+	+	NR	NR	NR	NR	NR	NR
Joint anomalies	-	+	+	NR	NR	NR	NR	NR	NR
<b>Cardiac anomaly</b>	-	-	-	ASD	VSD	NR	NR	bicuspid aortic valve	truncus arteriosus
<b>Skin (pigmentation) anomaly</b>	+	+	NR	NR	NR	NR	NR	NR	NR
<b>Hearing loss</b>	Mixed	-	-	NR	NR	NR	NR	NR	NR
<b>Ocular problems</b>	+1	+1	-	NR	+5	NR	NR	NR	+4

Clinical findings	Present study			Literature					
	Patient 1	Patient 2	Patient 3	4 <sup>1</sup>	5 <sup>13</sup>	6 <sup>5</sup>	7 <sup>16</sup>	8 <sup>16</sup>	9 <sup>19</sup>
<b>Gender</b>	Female	male	female	female	male	male	male	male	female
<b>Deletion 3q</b>	q23q24**	q23q25	q23q25	q23q25	q23q26	q25	q25.1q26.1	q25	q25
<b>Biometry</b>	-	+	+	-	NR	-	-	NR	NR
IUGR	+	+	+	-	+	-	-	NR	NR
Postnatal GR	+	+	+	-	NR	-	-	NR	NR
Microcephaly	+	+	+	-	NR	-	-	NR	NR
<b>Intellectual disability</b>	mild	+	+	+	severe	+	+	moderate	+
<b>Facial dysmorphism</b>	+	+	+	+	-	-	-	-	-
BPES	+	+	+	+	+	+	+	+	+
Upslanted palpebral fissures	+	+	+	+	+	+	+	+	+
Unusually shaped eyebrows	+	+	+	+	+	+	+	+	+
Coarse facies	+	+	+	+	+	+	+	+	+
Prominent nose	+	+	+	+	+	+	+	+	+
Ear anomalies	+	+	+	+	+	+	+	+	+
Macrostomia	+	+	+	+	+	+	+	+	+
Full lips	+	+	+	+	+	+	+	+	+
<b>Hypogonadism</b>	NR	NR	NR	NR	NR	NR	NR	NR	NR
<b>Genital anomalies</b>	-	-	NR	NR	NR	micropenis, hypospadias	-	NR	NR
<b>Skeletal anomalies</b>	-	+	+	+	+	+	+	+	+
Digit anomalies	-	+	+	+	+	+	+	+	+
Joint anomalies	-	+	+	+	+	+	+	+	+
<b>Cardiac anomaly</b>	-	VSD	VSD	-	NR	-	-	NR	NR
<b>Skin (pigmentation) anomaly</b>	NR	NR	NR	NR	NR	NR	NR	NR	NR
<b>Hearing loss</b>	+	NR	NR	NR	NR	NR	NR	NR	NR
<b>Ocular problems</b>	+	+	+	+	NR	NR	NR	NR	NR

a: tapering fingers; b: camptodactyly/clinodactyly/syndactyly; c: metatarsus adductus; d: arachnodactyly; e: flexion deformities of the fingers; f: brachydactyly; g: thickened interphalangeal joints; h: recessed 4<sup>th</sup> toes 1: refraction error; 2: strabismus; 3: optic nerve atrophy/hypoplasia; 4: microphthalmia; 5: iris coloboma. VSD: ventricle septum defect. MI: mitral valve insufficiency, TI: tricuspid valve insufficiency \*molecularly defined \*\* FISH + marker analysis. Other deletions are cytogenetically detected. NR= not reported. IUGR: intrauterine growth retardation. GR: growth retardation.



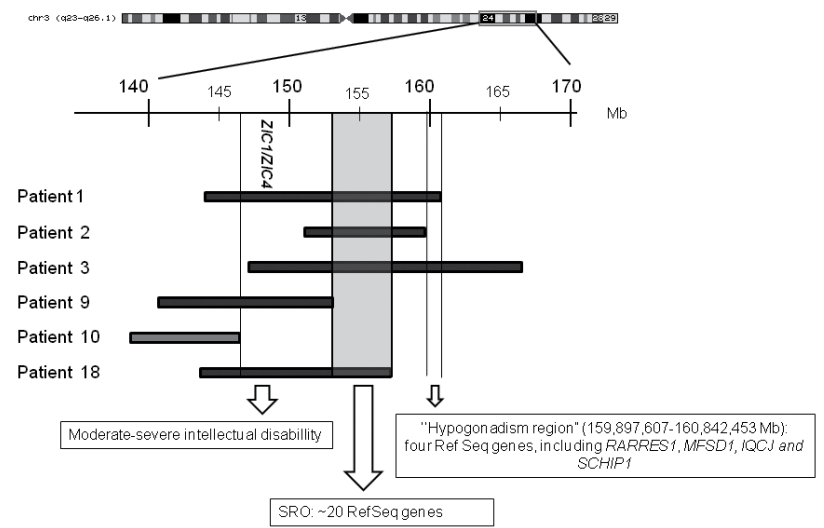
**Figure 2** Schematic overview of the deletions of patients 1-3 and overlapping deletions in 16 previously reported patients



Light grey bars indicate deletions detected by conventional cytogenetics. The grey bar indicates a deletion delineated by FISH and marker analysis (patient 10). The dark grey bars indicate deletions delineated by genome wide array analysis (patients 1-3, 9 and 18). The relative positions of the genes *FOXL2*, *ZIC1* and *ZIC4* are indicated.

overlapping deletion who do not show the clinical phenotype of BPES and thus, are the most suitable candidates for phenotype-genotype comparison with the present 3 patients, though comparison of the phenotypes is also hampered due to limited description of clinical features of patients 15-19. These 8 patients (1-3 and 15-19) with deletions located *distally* from the BPES region in 3q22.3 share several traits, including ID, prominent nose, genital anomalies and/or hypogonadism and digital anomalies. Genotype-phenotype comparison of the patients with a more precise determination of the breakpoints by genome wide array analysis or FISH and marker analysis (patients 1, 2, 3, 9, 10 and 18) documented that the overlapping deleted region of patients 1, 3 and 18, who have more severe developmental delay than patients 2 and 9, comprises the genes *Zinc finger protein of cerebellum, 1*

**Figure 3** Schematic overview of the four deletions identified and delineated by genome-wide array analysis and one deletion that was further delineated with FISH techniques and DNA marker analysis



Regions that might be associated with a more severe developmental delay and hypogonadism are indicated by vertical lines. The relative positions of the genes *ZIC1* and *ZIC4* are indicated. SRO: shortest region of overlap.

(*ZIC1*; OMIM\*600470) and *Zinc finger protein of cerebellum, 4* (*ZIC4*; OMIM \*608948) (Fig. 3). Patient 10 was too young age to assess the severity of her developmental delay. Interestingly, in patients with a deletion of 3q24, heterozygous loss of *ZIC1* and *ZIC4* has been reported to be the cause of Dandy-Walker malformation, which often is accompanied by developmental delay.<sup>21</sup> Moreover, the *Zic* family of zinc-finger proteins is thought to play a crucial role in neural development.<sup>22</sup> In most patients results of brain imaging were not reported, therefore structural brain anomalies in these patients, including patients 1, 3 and 18, are not excluded. In one of the previously reported patients with an overlapping deletion (patient 19) a Dandy-Walker malformation was reported. However, since this deletion is not molecularly defined, it is unclear whether it encompasses *ZIC1* and/

or *ZIC4*. Though this is speculative, molecular delineation might show involvement of *ZIC 1* and/or *ZIC4* in the deletion of this patient.

The region of overlap of the molecularly defined deletions of patients 1, 2, 3, 9 and 18 contains approximately 20 genes. None of these genes has previously been associated with ID. This region comprises one OMIM disease related gene, *solute carrier family 33, member A* (*SCL33A1*; OMIM\*603690), involved in autosomal dominant spastic paraplegia 42. In a single family with autosomal dominant inherited spastic paraplegia haploinsufficiency of *SCL33A1* was reported to be the cause of spastic paraplegia with a variable presentation in onset and severity, and incomplete penetrance.<sup>23</sup> Clinical signs of spastic paraplegia were not noticed in patients 1,2, 3, 9 and 18, neither in the 10 patients (patient 4, 11-19) with overlapping deletions. This might be explained by variable expression of the phenotype and/or incomplete penetrance as seen in the family reported by Lin et al.

Hypogonadism was exclusively observed in patients 1 and 3 and not reported in patients with overlapping deletions. Only taking into account the molecularly defined deletions, the unique region of overlap in patients 1 and 3 (159,897,607-160,842,543 Mb) comprises four known genes: *retinoic acid receptor responder 1* (*RARRES1*; OMIM\*605090), *major facilitator superfamily domain containing 1* (*MFSD1*), the *IQ motif containing J* (*IQCJ*), and the *Schwannomin interacting protein 1 gene* (*SCHIP1*; OMIM\*611622) (Fig. 3). None of these genes is likely to be directly involved in hypogonadism. However, *MFSD1*, *IQCJ* and *SCHIP1* fusion genes are expressed in the hypothalamus and haploinsufficiency of these genes therefore might hypothetically influence thalamic hormonal regulation, including the gonadotropin-releasing pathway. Underreporting of hypogonadism in patients with an overlapping deletion cannot be excluded, because it can easily be missed, especially before puberty. In males hypogonadism can present or be associated with micropenis and/or hypospadias, as seen in patient 16, but both traits can also have another origin.

Non penetrance of hypogonadism in patients with overlapping deletions and/or a coincidental occurrence of hypogonadism in patients 1 and 3 might be other explanations for the exclusive presentation of hypogonadism in patients 1 and 3. Patient 1 illustrates that it is important to search for signs of hypogonadism and to subsequently perform hormonal evaluation when indicated. Adequate treatment can prevent secondary complications like osteoporosis and cardiovascular diseases.

Patient 3 was previously reported as the second patient with Wisconsin syndrome on account of clinical similarity to a patient with Wisconsin syndrome, discovered in 1976 by John M. Opitz and later described by Cohen.<sup>2,20</sup> The Opitz patient reported by Cohen had craniosynostosis, ID, upslanting palpebral fissures, unusual shaped eyebrows, a prominent and coarse nose, microtia, and short fourth metatarsals

with recessed fourth toes. Karyotyping in 1976 showed normal results. However, at that time resolution was limited, so the presence of a small interstitial deletion could not be excluded. If, however, we assume that patient 3 is indeed a second patient with Wisconsin syndrome the other patients with an interstitial 3q deletion distal to *FOXL2* might also be considered as Wisconsin syndrome patients. These patients lack the short fourth metatarsals with recessed fourth toes and also the presence of craniosynostosis cannot be established, though patient 1 has an abnormal shape of her skull which is likely the result of abnormal closure of sutures. Further cases should provide evidence that Wisconsin syndrome is indeed caused by interstitial 3q deletions.

### Acknowledgements

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# 3.1.2

## **Clinical and molecular characterization of two patients with a 6.75 Mb overlapping deletion in 8p12p21 with two candidate loci for congenital heart defects**

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## Abstract

Clinical and molecular characteristics of two patients with a 6.75 Mb overlapping interstitial deletion in the 8p12p21 region are described and compared with previously reported cases with an overlapping deletion. The most common characteristics of interstitial deletions of proximal 8p are developmental delay, postnatal microcephaly and growth retardation. Other frequently reported findings are hypogonadism associated with haploinsufficiency of *GNRH1* and ocular problems. Congenital heart anomalies are also common and might at least to some extent be due to haploinsufficiency of *NKX2-6* or *NRG1*. The aforementioned clinical characteristics should be considered in the care of patients with a proximal interstitial 8p12p21 deletion.

## Introduction

Since the eighties of the last century the terminal 8p- syndrome was proposed as a new clinical entity with pre- and postnatal growth retardation, intellectual disability (ID), microcephaly, epicanthal folds, low-set and malformed ears, thin lips, micrognathia, short neck, wide-set nipples and congenital heart defect as reviewed by Tsukahara et al. in 1995.<sup>1</sup> Since then, the number of patients with a deletion of the short arm of chromosome 8 has increased progressively along with the improved ability of molecular techniques to identify small chromosomal aberrations.<sup>2,3</sup> Although most cases are terminal deletions encompassing the 8p23 region, several 8p syndromes associated with deletions in different regions on 8p have been reported.<sup>1,3-6</sup> It has been proven difficult to link the separate clinical features to specific chromosomal locations since the various different regions were just partly overlapping and not highly specific.<sup>2,3,7,8</sup> Moreover, so far only one reported case has been studied by molecular cytogenetic techniques which provided more accurate boundaries of the deletion.<sup>8</sup> The critical deleted region responsible for the clinical features observed in patients with a terminal deletion in 8p was initially assigned to the region between 8p21.3 and 8p23, because of absence of clinical features such as growth retardation, microcephaly and congenital heart defect in patients with a very distal 8p deletion (8p23-8pter).<sup>1,6</sup> However, several phenotypic features of this so called terminal 8p- syndrome were also described in patients with a proximal interstitial deletion not encompassing this 'critical region', for example in a patient with a deletion in 8p11.21p11.23.<sup>1</sup> More recently, the chromosome 8p23 deletion syndrome was outlined as a distinct syndrome including mild to severe ID, microcephaly, growth retardation, congenital heart defect and facial anomalies including micrognathia and low-set ears, the same major features as were previously attributed to the clinical entity terminal 8p- syndrome.<sup>3,4,7</sup> Other reported findings are behavioural disturbance, genitourinary anomalies and congenital diaphragmatic hernia.<sup>3,4,9-11</sup> Critical regions for some features have been delineated, such as heart defects assigned to 8p23.1, encompassing the *GATA4* gene<sup>3,6,7,9,12</sup> and *GNRH1* in 8p21.2 as a candidate gene for hypogonadism.<sup>8</sup>

To further delineate the phenotypic spectrum of interstitial deletions proximal to 8p23, we studied the clinical and molecular characteristics of two additional patients with overlapping interstitial 8p12p21 deletions and compared these with 19 previously published cases with an 8p deletion.



## Patients and methods

### Patient 1

This male was born at 40 weeks of gestation with a birth weight of 3,050 g (10<sup>th</sup>-25<sup>th</sup> centile). He was the first child of non-consanguineous parents. On the sixth day he was referred to the paediatrician because of feeding difficulties. An echocardiography, performed because of a heart murmur, showed a perimembranous ventricle septum defect (VSD) which was surgically corrected at the age of one month. Magnetic Resonance Imaging (MRI) of the brain at the age of 11 weeks showed hypoplasia of the corpus callosum and hypomyelination. At nine months of age he was referred to the clinical geneticist because of the aforementioned congenital malformations. By that time his length was 70 cm (25<sup>th</sup> centile), his weight was 7,200 g (2<sup>nd</sup> centile) and his head circumference was 43 cm (5<sup>th</sup>-10<sup>th</sup> centile). He had a prominent metopic suture, but craniosynostosis was excluded with a computer tomography (CT) -scan of the skull. He had a hypospadias. Facial dysmorphic features included upward slanting palpebral fissures, long eye lashes, small arched eyebrows, inverted epicanthal folds, bilateral malformation of the upper helices, a small right auricular pit, a long and smooth philtrum, thin lips and down turned corners of the mouth (Figs. 1 A-B). At 18 months of age head circumference had declined to the 2<sup>nd</sup> centile (45.5 cm) and length to the 0.6<sup>th</sup> centile (74 cm). A neuropsychological test showed a developmental delay of 2-5 months. An ophthalmologic examination revealed astigmatism. A hearing test showed bilateral sensorineural hearing loss of 70 decibel. Renal ultrasound did not show any abnormalities.

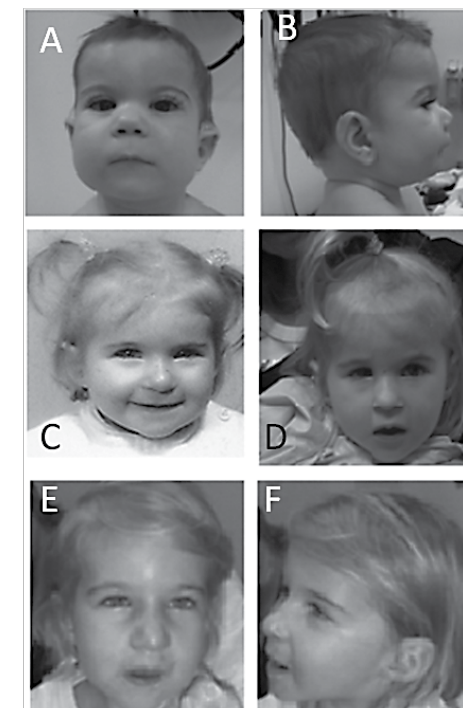
### Patient 2

This female was born at 42 weeks of gestation with a birth weight of 3,530 g (25<sup>th</sup>-50<sup>th</sup> centile). She was the second child of healthy non-consanguineous parents and had a healthy older sister. The amniotic fluid was meconium stained and she had a difficult start (Apgarscores 3 and 6 after 1 and 5 minutes respectively), but she quickly recovered. In the neonatal period she was hypotonic and had a gastroesophageal reflux. After four months very little eye contact and a high hypermetropia without structural eye abnormalities was noticed. At that moment length was 60.5 cm (25<sup>th</sup> centile), weight was 6,345 g (2<sup>nd</sup> centile) and head circumference was 39 cm (0.6<sup>th</sup> centile). At the age of one year further genetic diagnostic evaluation was performed because of developmental delay. Basic metabolic examination showed no abnormalities and a cerebral MRI did not reveal any structural brain malformations. At the age of 22 months she was referred to the clinical geneticist. The psychomotor development was delayed with standing independently at 21 months and she was speaking seven to eight words at that time. Upon physical examination height had declined to the 2<sup>nd</sup> centile (75 cm) and

head circumference to below the 0.6<sup>th</sup> centile (42 cm). She had mild facial dysmorphic features, including long eye lashes, narrow palpebral fissures, full hooked nose, broad dental gums and thin upper lip (Figs. 1 C-F). She also had broad thumbs and halluces, similar to her father.

**Figure 1** Photographs of two patients with a 8p12p21 deletion.

**A-B** Patient 1 at 9 months of age, showing long dark eye lashes, arched eyebrows, inverted epicanthal folds, a long and smooth philtrum, thin lips, downturned corners of the mouth and over folded upper helices of the ears. **C-F** Patient 2 at age of 12 months (C), 20 months (D) and 33 months (E-F), showing mild facial dysmorphic features, including thin upper lip, a full hooked nose and narrow palpebral fissures.



### Cytogenetic and FISH analysis

Cytogenetic analysis and Fluorescence In Situ Hybridisation (FISH) analysis was performed on cultured peripheral blood lymphocytes according to routine procedures.

For confirmation of the deletions detected by genome wide array analysis and for segregation analysis in their parents, region specific FISH analyses were performed using BAC-probes RP11-531L22 (8p21.2), RP11-615F9 and RP11-14D16 (8p21.1), RP11-139G09, RP11-317 J08 and RP11-724C15 (8p12).

### MLPA analysis

The subtelomeric regions were screened for imbalances by Multiplex ligation-dependent Probe Amplification (MLPA) analysis using commercially available kits (P036D and P070, MRC-Holland, the Netherlands) to test the patients' DNA samples. DNA was isolated from blood samples according to standard procedures.

### SNP array analysis

Genome wide Single Nucleotide Polymorphism (SNP) array analysis performed on DNA with the Affymetrix 250k SNP array platform according to the standard Affymetrix GeneChip protocol (Affymetrix, Inc., Santa Clara, CA, USA). Copy number estimates were determined using the updated version 2.0 of the CNAG (Copy Number Analyzer for Affymetrix GeneChip mapping) software package.<sup>13</sup> The normalized ratios were subsequently analyzed for genomic imbalances by a standard Hidden Marker Model (HMM), essentially as described before.<sup>6</sup>

## Results

### Clinical features

Clinical features of the two patients are shown in Table 1 and compared with those observed in 19 patients with an overlapping deletion previously reported in literature.

### Routine cytogenetic and molecular findings

Patient 1 had a normal male karyotype and 22q11.2 FISH analysis revealed a normal pattern. Patient 2 had a normal female karyotype, she did not have a 22q11.2 deletion upon FISH analysis and fragile X syndrome as well as Angelman syndrome were excluded by the respective molecular tests. Subtelomeric MLPA analysis did not reveal any imbalances in either patient one or patient two.

### Genome wide SNP array analysis

SNP array analysis revealed a significant deletion in 8p in both patients (schematically depicted in Figure 2). In patient 1, an interstitial 14.1 Mb loss was found in 8p21.3p12 (23.3-37.4 Mb; USCS genome browser build March 2006): 46 XY.arr snp 8p21.3p12 (SNP\_A-1870401→SNP\_A-2132186)x1 dn. The flanking normal SNP probes being SNP\_A-1860990 (distal) and SNP\_A-1937102 (proximal; centromeric). This loss was

in retrospect cytogenetically visible. In patient 2, an interstitial 6.75 Mb loss in 8p21.2p12 was identified (24.5-31.3 Mb; USCS genome browser build March 2006): 46,XX.arr snp 8p21.2p12(SNP\_A-2078711→SNP\_A-2226461)x1 dn. The distal and proximal (centromeric) array targets with normal ratios being SNP\_A-1936044 and SNP\_A-1885633, respectively. Region specific FISH analysis in the patients confirmed these losses and subsequent analysis in both parental couples gave normal results, suggesting a *de novo* origin of the deletion in both patients. The deleted region of patient 1 completely overlaps the deleted region of patient 2. In this overlapping region of 6.75 Mb more than 40 genes are localized. The distal, non-overlapping deleted region of patient 1 harbours six known genes, including *NKX2-6*. In the proximal non-overlapping region 15 genes including *NRG1* are localized.

## Discussion

Here two patients with an interstitial deletion of 8p12p21 are reported and compared with 19 previously reported cases with an overlapping deletion. Noteworthy, in the previously reported cases, except for the patient reported by Klopocki *et al.*<sup>8</sup> the deletions were identified by conventional cytogenetic studies and thus breakpoint delineations were not as precise as in patients 1 and 2 in whom the deletions and the breakpoints were delineated by genome wide SNP array analysis. This hampers exact genotypic comparison between the present and previously reported patients. Corresponding clinical features in the total number of 21 patients were psychomotor retardation (21/21), pre- and postnatal growth retardation (respectively in 12/21 and 17/21) and microcephaly (14/21) (Table 1). Patients 1 and 2 did not show evident facial resemblance (Figs. 1 A-B and 1 C-F). Facial features commonly described in patients with overlapping deleted regions were external ear anomalies (14/21), hypertelorism (7/21), epicanthal folds (11/21), micrognathia (9/21), short nose (5/21), small mouth (5/21) and thin lips (5/21). Some of these features were also noted in patient 1. Patient 2 showed remarkable facial resemblance to patient 13<sup>8</sup> with narrow palpebral fissures and thin upper lip. Cardiac anomalies, genital anomalies and ocular problems are frequently reported features, respectively present in 8, 14 and 9 out of the total number of 21 cases. Reported cardiac anomalies comprise pulmonary valve stenosis, atrio-ventricular septal defects, persistent left superior vena cava, hypoplastic right ventricle, subaortic stenosis and mitral valve insufficiency. In patients with an overlapping deletion encompassing the 8p23 region, a higher prevalence of congenital heart malformation was seen (5/7 versus 3/14). This is in agreement with previous studies that localized the critical region for heart defects, in a region encompassing the *GATA binding protein 4* (*GATA4*) (MIM 600576), on 8p23.1.<sup>3, 7, 12</sup> However, congenital heart defects were also reported in

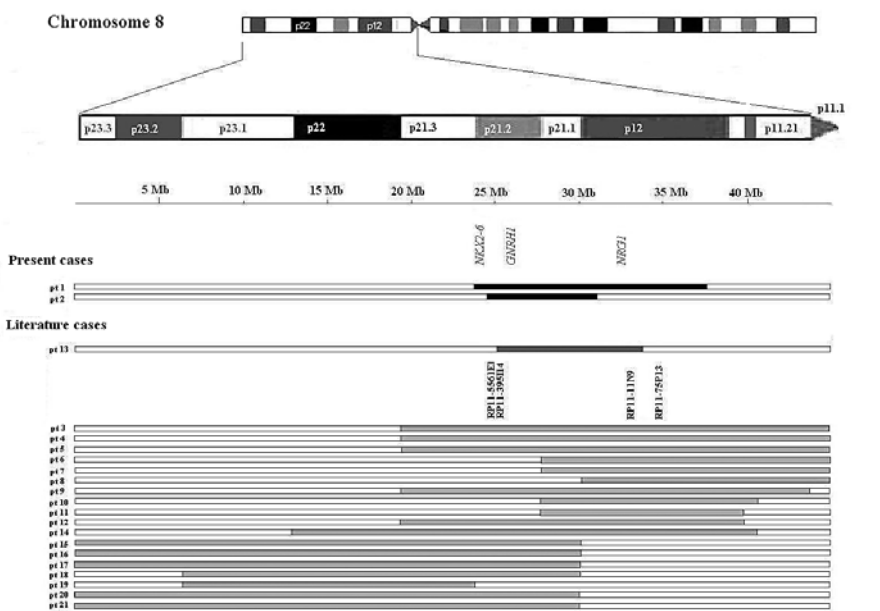
Table 1 Clinical features of patients 1 and 2 and previously reported patients with an overlapping chromosome 8p deletion											
Case Gender	Present		3 <sup>21</sup> F	4 <sup>18</sup> F	5 (sib of case 4) <sup>18</sup> M	6 <sup>19</sup> F	7 (sister of case 6) <sup>19</sup> F	8 <sup>22</sup> M	9 <sup>5</sup> M	10 <sup>23</sup> M	11 <sup>20</sup> M
	Patient 1 M	Patient 2 F									
Deletion 8p	p12-21.3	p12-21.2	p11-p21	p11-p21	p11-p21	p11.1-p21.1	p11.1-p21.1	p11.1-p12	p11.2-p21	p11.22-p21.1	p11.23-p21.1
Prenatal growth retardation	-	-	-	+	+	+	+	+	-	+	+
Postnatal growth retardation	+	+	+	+	+			+	+	+	+
Microcephaly	+	+	+	+	+				-	+	-
ID/DD	+	+	+	+	+	+	+	+		+	+
Neurological features											
Neuropathy	-	-		+		+	+				+
Cerebral MRI abnormality	+a/b	-									
Facial dysmorphism											
Hypertelorism			+								+
Epicanthal folds	+		+							+	+
Short nose											
External ear anomalies			+	+	+	+	+		+		+
Micrognathia			+	+	+						+
Small mouth											+
Thin lips	+	+									
Upslant palp fissures	+					+	+				
High arched palate			+							+	
Cardiac anomaly	+	-	-					-			+
Genital anomaly	+		+	+	+			+	+	+	+
Ocular abnormality	+	+	+			+	+	+			+
Auditory problems	+	-			+						
Spherocytosis	-	-		+	+	+	+	+		+	+

Case Gender	12 <sup>1</sup> F	13 <sup>8</sup> F	14 <sup>12</sup> M	15 <sup>2</sup> M	16 <sup>2</sup> M	17 <sup>24</sup> M	18 <sup>25</sup> M	19 <sup>26</sup> F	20 <sup>2</sup> M	21 <sup>2</sup> M	Total
Deletion 8p	p11.23-21.3	p12-p21.2	p12-p22	p21-p23	p21-p23	p21-p23	p21.1-p23.1	p21.3-23.1	p21-pter	p21-pter	
Prenatal growth retardation	+	-	-	+	-	-	+	-	+	+	12/21
Postnatal growth retardation		+	+	+	+	+	+	+	+	-	17/21
Microcephaly	-	+	+	+	+	-	+1	+	+	+	14/21
PMR/delayed development	+	+	+	+	+	+	+	+	+	+	20/21
Neurological features											
Neuropathy		+a/b/c		-	-						4/21
Cerebral MRI abnormality			+a/c								3/21
Facial dysmorphism											
Hypertelorism	+				+	+		+	+	+	7/21
Epicanthal folds	+			+	+	+		+	+	+	11/21
Short nose	+		+	+	+	+		+	+	+	5/21
External ear anomalies	+		+	+	+	+		+	+	+	14/21
Micrognathia	+					+		+	+	+	9/21
Small mouth	+								+	+	5/21
Thin lips	+	+									5/21
Upslant palp fissures											4/21
High arched palate	+							+	+	-	6/21
Cardiac anomaly	+	-	-	+	+	-	+	-	+	+	8/21
Genital anomaly	-	-	+	+	+	-	+		+	+	14/21
Ocular abnormality		+	+								9/21
Auditory problems											2/21
Spherocytosis	-	-					-				7/21

a: corpus callosum agenesis b: white matter hypoplasia c: dilated ventricles; 1: primary microcephaly;

?: not reported whether primary or secondary microcephaly

**Figure 2** Schematic overview of deleted regions on chromosome 8p in patients 1 and 2 and previously reported patients. The relative positions of *NKX2-6*, *GNRH1* and *NRG1* as well as the BAC array clones are indicated for clarity



patient 1 and two other patients with more proximal deletions (patient 11 and 12), suggesting other, more proximally located loci for heart defects as well. The deletion of patient 1 contains the gene *NK2 transcription factor related, locus 6* (*NKX2-6*) (MIM 611770) in 8p21.2. *NKX2-6* is one of several genes that are co-expressed with the gene *T Box-1* (*TBX1*) (MIM 602054) in the second heart field. *TBX1* is associated with velo-cardio-facial syndrome and is required for the development of the cardiac outflow tract, right ventricle and part of the atria. *Tbx1* knockout mice embryos have major cardiac outflow tract defects. *NKX2-6* is significantly down regulated in the second heart field of *tbx1* knockout mice embryos.<sup>14</sup> This suggests that *TBX1* enhances the expression of *NKX2-6*. The role of *NKX2-6* in cardiac outflow tract development is further supported by the finding that a homozygous missense mutation in *NKX2-6* was found in one large consanguineous family with persistent truncus arteriosus in six family members, which is due to failure of septation of the

cardiac outflow tract.<sup>14, 15</sup> The involvement of *NKX2-6* in the heart defects of patients with a deletion of 8p21 seems less likely, because of a heterozygous loss of this gene. However in the original report, only partial loss of activity of *NKX2-6* was shown by studying functional properties of the missense mutation.<sup>15</sup> So based on this, it is not ruled out that haploinsufficiency might contribute to the origin of heart defects. Another candidate gene for congenital cardiac malformations is the gene *Neuregulin-1* (*NRG1*) (MIM 142445) in 8p12. *NRG1* is involved in two different aspects of cardiac development, namely trabeculation (development of the finger-like extensions of the heart's wall) and valvuloseptal formation. *Nrg1* null mutations in mice were shown to result in an embryonic-lethal cardiac defect.<sup>16, 17</sup> *NRG1* was also deleted in patient 13<sup>8</sup>, though she did not have a heart defect. This might be explained by non-penetrance or a multifactorial cause of congenital heart defects. Furthermore, *NRG1* is shown to have many functions during neural development, including stimulation of neuronal migration and differentiation.<sup>16, 17</sup> Haploinsufficiency of *NRG1* could therefore also play a role in the occurrence of peripheral neuropathy that was reported in patient 13.<sup>8</sup> However, our patient 1 did not show signs of neuropathy at 18 months of age. Interestingly, neuropathy was also reported in patients 4<sup>18</sup>, 6 and 7<sup>19</sup> and 11<sup>20</sup>, in whom the proximal cytogenetic breakpoints of the deletion are in 8p11, suggesting that *NRG1* is deleted.

Genital anomalies in the patients with proximal 8p deletions include hypospadias, cryptorchidism, micropenis and more generally, hypogonadism. Genital anomalies were only noted in patient 1. However, in females hypogonadism might not present before puberty as seen in patients 4 and 11 when hypogonadism manifested in puberty by primary failure of sexual development and primary amenorrhea.<sup>18, 21</sup> Almost all patients with genital anomalies had a deletion of 8p21.2 which harbours gonadotropin-releasing hormone 1 (*GNRH1*) (MIM 152760), the gene that is suggested to be associated with genital anomalies.<sup>8</sup>

Mild ocular abnormalities were seen in both of our patients. Patient 1 had astigmatism and patient 2 had hypermetropia. Reported ocular problems in literature are very heterogeneous and vary from aspecific or mild (for example strabismus, hypermetropia and myopia) to more severe disorders like retinal dysplasia and iris coloboma in patient 11<sup>20</sup>, and hypoplastic papillae in patient 13.<sup>8</sup> It seems that ocular problems occurred more often in the more proximal deletions encompassing 8p11p12, whereas ocular problems were not reported in deletions distally from 8p21. This suggests that genes located in the more proximal deleted region could be implicated in (severe) ocular problems, although we were not able to identify a candidate gene in this region. In 2006 Klopocki et al.<sup>8</sup> suggested *EXTL3* (MIM 605744), which is located on region 8p21.1, as a candidate gene for the ocular abnormalities in their patient (corresponding to patient 13) and in the case reported by Okamoto et al. (corresponding to patient 11). *EXTL3* is thought to be involved in

axonal guidance in the optic tract. Disruption of this gene could possibly result in ophthalmological problems related to distortion of the optical nerve and related structures (i.e. retinal dysplasia and papillar hypoplasia, respectively reported in patient 11 and 13).<sup>8</sup> However a causal relation between disruption of this gene and the occurrence of other reported, more aspecific, ocular abnormalities such as strabismus and hypermetropia seems very unlikely.

Structural brain malformations are rare. In patient 1, however, hypoplasia of the corpus callosum and hypomyelinisation were noted. Similar abnormalities were also reported in patients 13<sup>8</sup> and 14<sup>12</sup>. Since cerebral imaging was not reported in every patient, brain abnormalities might have gone unnoticed. Seizures are also rarely reported in patients with 8p deletions and were not present in our patients either. Sensorineural hearing loss is present in patient 1. Hearing loss is rarely reported in the published cases. One previously reported patient had a conductive hearing loss due to congenital stapedial fixation.

In summary, we conclude that interstitial deletions of the proximal short arm of chromosome 8 are characterized by psychomotor retardation, postnatal microcephaly and growth retardation. The facial features vary considerably which hamper the recognition of patients on a characteristic facial appearance. Frequently reported features are hypogonadism, probably associated with haploinsufficiency of *GNRH1*, cardiac anomalies, which might be associated with haploinsufficiency of *NKX2-6* and *NRG1*, and ocular anomalies. Therefore, these features should be considered in clinical management of patients with proximal 8p deletions.

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Patient consent was obtained from the patient's parents for publishing patient pictures in fig. 1.

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# 3.2.1

## Identification of *ANKRD11* and *ZNF778* as candidate genes for autism and variable cognitive impairment in the novel 16q24.3 microdeletion syndrome

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## Abstract

The clinical utilization of array comparative genomic hybridization in the evaluation of patients with multiple congenital anomalies and/or intellectual disability has recently led to the discovery of a number of novel microdeletion and microduplication syndromes. We present four male patients with overlapping molecularly defined *de novo* microdeletions of 16q24.3. Clinical features observed in these patients include facial dysmorphisms comprising prominent forehead, large ears, smooth philtrum, pointed chin and wide mouth, variable cognitive impairment, autism spectrum disorder, structural anomalies of the brain, seizures, and neonatal thrombocytopenia. While deletions vary in size, the common region of overlap is only 90 kb and comprises two known genes, *Ankyrin Repeat Domain 11* (*ANKRD11*) (MIM 611192) and *Zinc Finger 778* (*ZNF778*), and is located approximately 10kb distally to *Cadherin 15* (*CDH15*) (MIM 114019). This region is not found as a copy number variation in controls. We propose that these patients represent a novel and distinctive microdeletion syndrome, characterized by autism spectrum disorder, variable cognitive impairment, facial dysmorphisms and brain abnormalities. We suggest that haploinsufficiency of *ANKRD11* and/or *ZNF778* contribute to this phenotype and speculate that further investigation of non-deletion patients who have features suggestive of this 16q24.3 microdeletion syndrome might uncover other mutations in one or both of these genes.

## Introduction

Whole-genome scanning technologies such as array comparative genomic hybridization (array CGH) and single nucleotide polymorphism oligonucleotide arrays (SNP array) have enabled the detection of submicroscopic chromosomal aberrations, which previously escaped detection by routine cytogenetic and molecular cytogenetic techniques.<sup>1-3</sup> These methods have proven invaluable in the elucidation of genomic regions associated with intellectual disability (ID) and/or congenital anomalies.<sup>4-8</sup> Several clinically distinct microdeletion and microduplication syndromes have been reported based on data derived from these techniques such as the 17q21.31 microdeletion syndrome<sup>5</sup> and the 1q41-1q42 microdeletion syndrome<sup>9</sup>, as well as microduplication syndromes involving 7q11.23<sup>10, 11</sup> and 17p11.2.<sup>12</sup> The phenotypic characteristics of microdeletion syndromes can be caused by haploinsufficiency of single genes, for example *TCF4* (MIM 602272) in Pitt-Hopkins syndrome,<sup>13</sup> *EHMT1* (MIM 607001) in 9q34.3 subtelomeric deletion syndrome<sup>14</sup> and either *CREBBP* (MIM 600140) or *EP300* (MIM 602700) in Rubinstein Taybi syndrome.<sup>15, 16</sup> The application of genome-wide array technologies with increasing density of probes has led to the identification and localization of several genes associated with developmental disorders or abnormal brain development<sup>9</sup> including *CHD7* (MIM 608892) in CHARGE syndrome<sup>17</sup> and *FOXG1* (MIM 164874) in congenital Rett syndrome.<sup>18</sup> Aberrations of chromosome 16q with clinical relevance have rarely been reported. Previous to the present report, interstitial deletions restricted to band 16q24.3 have not been described. There are only a few reports in the medical literature of patients with larger, cytogenetically visible deletions comprising this region, mostly due to an unbalanced complex chromosomal rearrangement.<sup>19-29</sup> In this study we aimed to characterize the clinical and molecular features of four patients with submicroscopic interstitial 16q24.3 microdeletions ascertained by genome-wide array analysis and to determine the shortest region of overlap (SRO) to identify candidate genes responsible for their overlapping phenotype.

## Patients and Methods

### Patient 1

Patient 1 was ascertained at age 22 years when SNP array was performed because of ID, autism spectrum disorder, dysmorphic features and congenital anomalies. He was born after an uncomplicated pregnancy, induced at 36 weeks gestation because of maternal preeclampsia. Growth parameters were appropriate for gestational age with a birth weight between the P50 and P75. Head circumference was not measured at birth. He was diagnosed with congenital hip dysplasia, but

had no other difficulties in the neonatal period. His psychomotor development was delayed. He walked independently at the age of 2,5 years and began using single words after age four years. At the age of three years he was referred to a pediatric neurologist, because of his developmental delay and epilepsy (absence and generalized epilepsy). Cerebral imaging with a computer tomography-scan (CT-scan) showed colpocephaly, hypoplasia of the corpus callosum and heterotopia. Vision and audiological evaluations were normal. Routine chromosome analysis showed a normal male karyotype. At the age of 22 years he was referred for reevaluation. He had a moderate ID and showed behavior consistent with autism spectrum disorder. A Dutch formal test for autism spectrum disorders (AVZ-R ("Autisme- en Verwante stoornissenschaal-Z-Revisie", 1999) showed borderline results for the diagnosis of an autism spectrum disorder. His epilepsy was under good control and medical treatment was gradually stopped. On physical examination he exhibited dysmorphic features, with high forehead, bitemporal narrowing, long palpebral fissures, large ears, smooth philtrum, wide mouth and micrognathia (figure 1 C-D). He had a kyphoscoliosis and strabismus. His height was 175 cm (3<sup>rd</sup>-10<sup>th</sup> centile), his weight was 64 kg (50<sup>th</sup> centile) and his head circumference was 59.5 cm (90<sup>th</sup> centile). Cardiac ultrasound revealed no abnormalities. Bone densitometry showed normal bone density. Genetic diagnostic evaluation with subtelomeric multiplex ligation-dependent probe amplification (MLPA, SALSA MLPA Kit P036, MRC Holland, Amsterdam, The Netherlands) was normal.

### Patient 2

A cursory analysis of patient 2 has been included in a large Canadian autism cohort study,<sup>30</sup> and here we provide a more detailed clinical and molecular description. Patient 2 was ascertained at age 3 years and 3 months when SNP array analysis was performed because of developmental delay, autism spectrum disorder and dysmorphic features. He was born at term after an uncomplicated pregnancy and delivered by Caesarean section on maternal indication. He was born with strikingly outstanding and asymmetric ears. On day three he developed low platelets. It was documented as caused by alloimmune idiopathic thrombocytopenic purpura (ITP) due to his mother's ITP. After treatment his platelets remained within the normal range. Psychomotor development was delayed. At 21 months of age he was not able to walk independently and used 3 single words with meaning. He was diagnosed with autism by formal tests, at age 2 years by ADOS-1 (Autism Diagnostic Observation Schedule) and at age 3 years and 4 months by ADI-R (Autism Diagnostic Interview Revised). Psychometric testing at age 6 years and 5 months showed average results for nonverbal intelligence; Naglieri Nonverbal Ability test (NNAT) score was at the P50 and Test of nonverbal intelligence (TONI-3) gave a

nonverbal IQ of 100, Preschool Language Scale 4 (PLS-4) showed that auditory comprehension was severely delayed (1<sup>st</sup> centile) and that expressive communication was moderately delayed (P4). On physical examination, he had a height of 94 cm (10<sup>th</sup>-25<sup>th</sup> centile) and weight of 13.5 kg (10<sup>th</sup>-25<sup>th</sup> centile) and a head circumference of 49.5 cm (50<sup>th</sup> centile). Facial dysmorphic features included frontal bossing, long palpebral fissures, wide mouth with full lips, long and smooth philtrum, anteverted nares, pointed chin and large ears (ears originally outstanding/cupped and asymmetric, now post surgical correction) (figure 1E). Diagnostic studies, including routine G-banded chromosome analysis, FISH for the Williams syndrome microdeletion, and molecular analysis for fragile X syndrome were negative. CT-scan of the brain was normal and examination by the ophthalmologist did not show any abnormalities.

### Patient 3

Patient 3 was ascertained at age 6 years 3 months when array CGH analysis was performed because of ID, dysmorphic features, and congenital anomalies. He was born at term by vaginal delivery after an uncomplicated pregnancy and his size was appropriate for gestational age. At delivery he was noted to have a skin rash and hepatosplenomegaly with thrombocytopenia. TORCH (toxoplasmosis, rubella, cytomegalovirus, herpes) infection was excluded and the organomegaly and thrombocytopenia resolved. He suffered a mild intracranial hemorrhage secondary to the thrombocytopenia, and was diagnosed with a ventricular septal defect (VSD), patent foramen ovale (PFO), cleft mitral valve and left cryptorchidism in the newborn period. VSD and PFO were repaired at age 3 months. Routine G-banded chromosome analysis was normal. At the time of the genetics consultation at age 6 years and 3 months he had been admitted to hospital for an acute parvovirus myocarditis and dilated cardiomyopathy with severely depressed biventricular function. Further evaluation revealed a history of developmental delay (he walked at age 3 years, and spoke his first words at 18-20 months), ID and febrile seizures. On physical examination he had a height on the 5<sup>th</sup> -12<sup>th</sup> centile and a head circumference on the 10<sup>th</sup> centile. Dysmorphic features that were observed included a triangular face with a high forehead and frontal bossing, arched eyebrows, large ears, smooth philtrum, wide mouth, pointed chin and high palate (figure 1 F-G). MRI of the brain revealed a posterior fossa arachnoid cyst, thinned corpus callosum, periventricular heterotopias, optic nerve hypoplasia, and evidence for an old left hemorrhagic parietal infarct. The heterotopias were unilaterally localized along the lateral margin of the trigone of the right lateral ventricle extending along the temporal horn of the right lateral ventricle. Ophthalmic examination revealed high myopia, intermittent exotropia, horizontal nystagmus, and possible right amblyopia. Audiogram revealed moderate to severe bilateral mixed sensorineural and conductive hearing



impairment. Complete blood count showed mild macrocytosis (MCV 92.4 fL; normal 76-90), with normal homocysteine and folate values. The cardiomyopathy and macrocytosis resolved, yet he developed complex partial seizures at age 7 ½ years with EEG showing a seizure focus of the left temporal region. He continued to make progress in school. He meets criteria for autism under the ADOS.

#### Patient 4

Patient 4 was ascertained at age 8 years and 10 months when array CGH analysis was performed because of psychomotor retardation, features of autism spectrum disorder and dysmorphic features. He was born at term after an uneventful pregnancy by a Caesarean section because of breech presentation. Birth weight was 3,850 grams (50<sup>th</sup>-90<sup>th</sup> centile). Birth length and head circumference are unknown. In the first weeks there were feeding problems, probably caused by poor sucking. There was no hypotonia. Psychomotor development was slightly delayed with independently sitting at 12 months of age and independently walking at the age of 18 months. Language development was delayed. Throughout childhood there were periods of obstipation. There were frequent ear infections. Hearing was tested normal. There is severe bilateral astigmatism. At the age of 7 years and 2 months verbal IQ was 81 and performatory IQ was 67 as assessed by the WISC III intelligence test. He was diagnosed with an Autism Spectrum Disorder (ASD)/Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS) by a child psychologist and psychiatrist. There are no signs of epilepsy. On physical examination he showed dysmorphic features with a high and broad forehead, mildly upslanting palpebral fissures, a double hair whorl, large ears, a preauricular tag and bilateral fusion of the central and lateral incisors (figure 1 H-I). His height was 126.3 cm (10<sup>th</sup>-25<sup>th</sup> centile), his weight was 23.7 kg (5<sup>th</sup>-25<sup>th</sup> centile) and his head circumference was 52.2 cm (50<sup>th</sup>-75<sup>th</sup> centile). Cardiac examination was normal. The left hand showed a single transverse palmar crease. Thumbs were slightly proximally placed. X-rays of the hands showed pseudo-epiphyses of the second metacarpals. Routine G-banded chromosome analysis showed a normal male karyotype. Molecular analysis for fragile X syndrome was negative. Complete blood count showed no abnormalities at the age of 8 years.

#### Methods

DNA was obtained from peripheral blood leucocytes and isolated according to standard procedures. In patient 1 and 2 and both parental couples a 500k SNP array analysis with the combined two-chip Affymetrix *Nsnp* and *Styl* GeneChip Human Mapping Commercial was performed according to the standard Affymetrix GeneChip protocol (Affymetrix inc, Santa Clara, California, USA). For CNV validation in patient 2, multiple SYBR Green-based qPCR assays were used (primer sequences

available upon request) to measure relative copy number in the patient and parents and controls between this chromosome 16q24.3 and a control region (*FOXP2*). The same approach was used to validate a *de novo* 500 kb CNV gain at 3p14.2 near the common fragile site *FRA3B* in patient 2. Subsequently, patient 2 was also tested on the Affymetrix 6.0 array.

In patient 3 and his parents a 244K oligo array analysis, according to the Agilent protocol (Agilent Technologies Inc., Santa Clara, CA, USA) was performed.

In patient 4 and his parents a 105K oligo array according to the Agilent protocol was performed (design ID 019015, Agilent Technologies Inc.).

## Results

### Clinical features

A summary of the clinical features of the four patients with a chromosome 16q24.3 microdeletion is shown in Table 1. All patients show a prominent forehead and large ears. Moreover, patients 1-3 share distinct other features (Figure 1A-G) including long smooth philtrum, broad mouth and pointed chin. These were not seen in patient 4 (Figure 1H-I). In addition to variable cognitive impairment, ranging from moderate ID to normal nonverbal IQ with moderate-severe speech delay, all four patients have either autistic features (patient 1) or met the diagnostic criteria for an Autistic Spectrum Disorder (ASD) (patients 2-4). Interestingly, brain abnormalities, including structural anomalies and neuronal migration disorders, were noticed in two of the three patients who underwent brain imaging (patient 1 and 3). Epilepsy and (transient) thrombocytopenia were each noticed in two out of the four patients. Nonspecific ocular problems were seen in three of the four patients. One patient, patient 3, with the largest deleted region, had a ventricular septal defect and cleft mitral valve.

### Molecular findings

The *de novo* CNV results observed in the four patients are summarized in Table 1 and the overlapping deletions at chromosome 16q24.3 are schematically shown in Figure 2. All deletions were mapped according to the USCS genome browser build May 2004.

In patient 1 an interstitial 378 kb loss was identified (87,65-88,03Mb): 46, XY, arr snp 16q24.3 (SNP\_A-1895824→SNP\_A-2058988)x1 dn.

In patient 2 an interstitial loss of 265 kb (87,80-88,06 Mb): 46,XY, 16q24.3 (SNP\_A-2223520 → SNP\_A-4274603)x1 dn was identified. Detailed analysis of the qPCR data in patient 2 revealed that the deletion does not extend to the last exon of *CDH15*. According to the assays the coordinates 87,788,807-87,788,909 (Build 36)



**Table 1** Clinical features of the four presented patients with a 16q24.3microdeletion

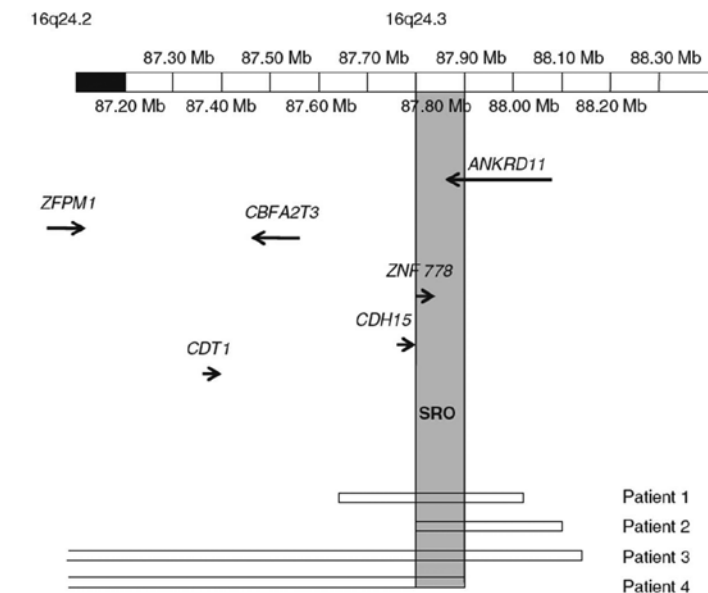
Patient	Patient 1	Patient 2	Patient 3	Patient 4	Total
<b>Size of 16q24 deletion</b>	378 kb	265 kb	2.07 Mb	1.1 Mb	90 kb overlap
<b>Mb positions</b>	87,65-88,03	87,80-88,06	86,06-88,13	86,79-87,89 Mb	SRO: 87,80-87,89 Mb
<b>Age at examination</b>	22y	3y, 3m	6y, 3m	8y, 10m	mean age 10y
<b>Cognitive impairment</b>	moderate ID	normal nonverbal IQ with moderate-severe speech delay	Moderate ID	borderline-normal verbal IQ mildly impaired nonverbal IQ	4/4
<b>Autism Spectrum Disorder</b>	borderline	+	+	+	4/4
<b>Height</b>	3-10th percentile	10-25th percentile	5th-12th percentile	10-25 <sup>th</sup> percentile	all (low) normal
<b>Head circumference</b>	90th percentile	50th percentile	10th percentile	50-75 <sup>th</sup> percentile	all normal
<b>Facial characteristics</b>					
High forehead	+	-	+	+	3/4
Frontal bossing	-	+	+	-	2/4
Bitemporal narrowing	+	-	-	-	1/4
Long oval face	+	-	-	-	1/4
Long palpebral fissures	+	+	-	-	2/4
Arched eyebrows	-	-	+	-	1/4
Large ears	+	+	+	+	4/4
Smooth philtrum	+	+	+	-	3/4
Broad mouth	+	+	+	-	3/4
Pointed chin	+	+	+	-	3/4
Micrognathia	+	+	-	-	2/4
High palate	+	-	+	-	2/4
<b>Congenital heart defect</b>	-	-	VSD, PFO and cleft mitral valve with severe mitral regurgitation	NT	1/2
<b>Structural brain malformation</b>	corpus callosum hypoplasia colpocephaly	-	corpus callosum hypoplasia dilated ventricles optic nerve hypoplasia	NT	2/3
<b>Neuronal migration disorder</b>	heterotopias	normal unenhanced CT cerebrum	perventricular heterotopias (unilateral)	NT	2/3
<b>Seizures</b>	absences and generalized	-	partial complex	-	2/4
<b>Ocular problems</b>	strabismus	-	high myopia astigmatism horizontal nystagmus	severe bilateral astigmatism	3/4
<b>Hearing loss</b>	-	-	mixed sensorineural and conductive hearing loss	-	1/4
<b>Skeletal anomalies</b>	kyphoscoliosis	-	-	-	1/4
<b>Genital anomalies</b>	-	-	unilateral cryptorchidism	-	1/4
<b>Hematologic disorder</b>	-	neonatal thrombopenia (resolved)	thrombopenia, macrocytosis (resolved)	-	2/4

NT= not tested

**Figure 1** Facial profiles of patients 1-4. **A-C:** Patient 1 at several ages from 6 years (A) to 22 years (B-C). **D-F:** Patient 2 at the age of 3 years (D) and at the age of 8 years (E-F). **G-H:** Patient 3 at the age of 7 years. **I-J:** Patient 4 at the age of 8 years and 10 months. Facial features comprise high forehead/frontal bossing and large ears (all patients), broad mouth, long smooth philtrum and pointed chin (patient 1-3). Note the change in facial phenotype in patient 1 at adult age, showing a long and oval face with full upturned nose, retrognathia and pronounced groove in the chin.



**Figure 2** Schematic overview of deleted regions on chromosome 16q24.3 in the presented patients.



All deletions were mapped according to the UCSC genome browser build May 2004. The relative positions of the genes of interest are indicated. The region of 90 kb overlap is demarcated with a grey zone. The deletions of patient 3 and 4 extend beyond the figure (Mb positions of proximal delineations: 86,06 and 86,79 respectively). The arrows indicate the direction in which the genes are transcribed.

are not deleted (results not shown). Patient 2 also carried a 500 kb *de novo* gain at 3p14.2. An interstitial loss of 2.07 Mb in chromosome band 16q24.2q24.3 was identified in patient 3 (86,06-88,13 Mb) (probe A\_16\_P40717088 → probe A\_16\_P20559418). In patient 4, an interstitial loss of 1,1 Mb in chromosome band 16q24.2q24.3 was detected (86,79-87,89 Mb) (probe A\_14\_P119277 → A\_14\_P111955).

In all four cases analysis of both parents showed normal copy numbers for the deleted regions, suggesting a *de novo* origin of the deletions. These four chromosome 16q24.3 deletion cases are the only cases that have been detected in a total cohort of more than 3000 patients with developmental delay and/or autism or congenital anomalies. The same region was interrogated over 1000 population controls<sup>31</sup> and found not to be copy number variable. *The Database of Genomics Variants*<sup>32</sup> shows

one CNV (Variation\_4018) at this site<sup>33</sup>, involving the first two exons of *ANKRD11* but not *ZNF778*, but it is not found in other studies.

## Discussion

We present detailed clinical and molecular features of four patients with micro-deletions within the chromosomal band 16q24.3. The four patients have autistic features or a diagnosis of ASD, variable cognitive impairment and facial dysmorphism, and share a 90kb overlapping region comprising two annotated genes, *Ankyrin Repeat Domain 11* (*ANKRD11*) and *zinc finger 778* (*ZNF778*) (Figure 2). The level of cognitive impairment that was observed in these four patients is considerably variable, ranging from moderate ID in patient 1 and 3 to a normal nonverbal IQ with moderate-severe speech delay in patient 2. Patient 2 showed a remarkable discrepancy between his verbal and nonverbal capacities in disadvantage of speech/auditory development. An opposite discrepancy in IQ levels, however less extreme, was reported in patient 4 who had a borderline-normal verbal IQ of 81 and a mildly impairment nonverbal IQ of 67.

Remarkably, both patients 1 and 3 have structural brain abnormalities, including hypoplasia of corpus callosum, colpocephaly/dilated ventricles, as well as periventricular heterotopias that might be causative for the epilepsy these two patients experienced. They share a deletion interval comprising *ANKRD11*, *ZNF778* and *CDH15*, suggesting that haploinsufficiency for one or more of these genes is involved in neuronal migration and causes the gray matter heterotopias in patient 1 and 3. The localization of the heterotopias in patient 1 was likely bilaterally, but no further details were available by cerebral imaging for this patient. The heterotopias in patient 3, however, were unilaterally localized which raises the possibility that this migrational defect was caused by an (in utero) acquired event rather than a genetic defect. Both patient 2 and 3 had temporary thrombocytopenia, although the neonatal thrombocytopenia in patient 2 was attributed to his mother's ITP. Nonspecific ocular problems were noted in three of the four patients. Features that were only observed in patient 3 (with the largest deleted region) were a congenital heart defect, unilateral cryptorchidism and sensorineural hearing impairment. Patient 2 also carried a 500 kb *de novo* gain at 3p14.2 at the *FRA3B* (MIM 601153) locus, which may also contribute to his phenotype, however patient 2 had no specific other features.

By molecular characterization of these four overlapping deletions in 16q24.3, two likely candidate genes for the observed autism phenotype with cognitive impairment were present in the SRO. A third gene, *Cadherin 15* (*CDH15*), is located just proximal to the SRO and is involved in the deletion of patients 1, 3 and 4 (Figure 2). *CDH15*

appears not to be involved in the deletion of patient 2. It is not excluded that a position effect or disruption of gene regulatory elements could create a functional null allele for *CDH15* also in this patient. The potential involvement of *CDH15* is of particular interest since nonsynonymous variants of this gene have been identified in a cohort of mentally disabled patients.<sup>34</sup> However the group of non-affected controls in this study was relatively small and segregation of the variants in unaffected family members has not been tested consistently or was not in agreement with the presumed pathogenicity of the detected variant. Of interest, a recently published genome wide association study among cases with ASD identified common genetic variants on 5p14.1 that are associated with susceptibility to ASD. The nearby genes *CDH9* (MIM 609974) and *CDH10* (MIM 604555) are also members of the Cadherin gene family involved in neuronal cell adhesion. This implicates a role of *CDH9* and *CDH10* and possibly other members of the Cadherin family in the pathogenesis of ASD by affecting the neuronal cell adhesion.<sup>35</sup>

Haploinsufficiency for either or both *ANKRD11* and *ZNF778* is an attractive explanation for the syndromic autism phenotype with variable cognitive impairment observed in the four patients. *ANKRD11* encodes a member of the family of ankyrin repeat containing cofactors that interacts with p160 nuclear receptor coactivators and histone deacetylases corepressors, and inhibits ligand-dependent transcriptional activation of target genes by nuclear receptors.<sup>36</sup> Therefore, haploinsufficiency of *ANKRD11* may disrupt transcription regulation. Disruption of epigenetic processes has been shown to play a role in the cause of ID. Examples are disruption of a methyl-CpG-binding protein caused by mutations in *MECP2* (MIM 30005) in Rett syndrome, mutations in *EMHT1* in the 9q subtelomere deletion syndrome and mutations in either *CREBBP* or *EP300* in Rubinstein-Taybi syndrome. In these cases ID might be caused by epigenetic effects on the expression of genes that are not themselves mutated.<sup>37</sup>

Interestingly, Barberic et al.<sup>38</sup> described a mouse mutant, named *Yoda*, with a missense mutation in the *Ankrd11* gene. Homozygous knock out *Yoda* mice are not viable and die during embryogenesis. This suggests that the gene has a crucial function in embryonic development. A major feature of heterozygous mutant mice is reduced bone mineral density. They also show cranio-facial abnormalities, like shortened snouts, wider skulls, deformed nasal bones and failure of the interfrontal suture to close. Body size is reduced and with ageing many *Yoda* mice developed kyphosis.<sup>38</sup> Mild kyphoscoliosis was seen only in the oldest patient (patient 1), however examination of bone density in this patient showed normal results. Based on *in silico* data *ANKRD11* is widely expressed, including in several brain regions (amygdala, parietal lobe, occipital lobe and cerebellum) and the heart (UCSC Genome Browser, Microarray Expression Data, Unigene).

The other gene localized in the SRO, *ZNF778*, encodes a member of the extensive

family of KRAB-domain zinc finger proteins. Based on the available *in silico* data, this gene is also expressed in several tissues, including the brain and the heart (Unigene). KRAB-domain zinc finger proteins are found in transcription regulatory complexes, which are directed to the regulatory elements of target genes through the C2H2 zinc finger domains that recognize specific DNA binding sites. Moreover, several human zinc finger genes have been associated with MR: *ZNF41* (MIM 314995), *ZNF81* (MIM 314998) and *ZNF674* (MIM 300573) with X-linked MR<sup>39-41</sup> and

*ZNF385B* (MIM 612344) with the 2q31.2 deletion syndrome.<sup>41</sup> Therefore, haploinsufficiency of *ZNF778* might also, or exclusively, be involved in the phenotype observed in the reported patients.

Table 2 provides an overview of these and other interesting genes that are involved in the non-overlapping deleted regions. Proposed functions, phenotype in knockout mice (if available), previously reported cases and the possible clinical correlation of haploinsufficiency of these genes with the observed phenotype in our patients are

**Table 2** Clinical features of patients 1 and 2 and previously reported patients with an overlapping chromosome 8p deletion

Gene (Mb positions)	Function	Mouse null	Mouse heterozygous	Previously reported cases	Possible clinical correlate
<i>ANKRD11</i> (87,86-88,08)	nuclear receptor mediated transcriptional regulation (inhibitory) <sup>36, 38</sup>	lethal <sup>38</sup>	reduced bone mineral density  craniofacial abnormalities  reduced body size <sup>38</sup>	Marshall et al. identified a 16q24.3 deletion encompassing <i>ANKRD11</i> in an ASD case <sup>30</sup>	kyphoscoliosis in patient 1, however he has a normal bone density  syndromic autism phenotype with variable cognitive impairment in patients 1, 2, 3 and 4
<i>ZNF778</i> (87,81-87, 82)	encodes a member of the KRAB domain containing zinc finger protein family, suggesting involvement in transcription regulation <sup>39-42</sup>	no data available	no data available	Several other human zinc finger genes have been associated with ID <sup>39-42</sup>	syndromic autism phenotype with variable cognitive impairment in patients 1, 2, 3 and 4
<i>CDH15</i> (87,77-87,79)	cell-cell adhesion in skeletal muscle and human brain <sup>34, 43</sup>	no data available	no data available	Bhalla et al. identified 4 nonsynonymous variants of <i>CDH15</i> in a cohort of patients with ID <sup>34</sup>	cognitive impairment in patient 1, 3 and 4
<i>ZFPM1 (FOG1)</i> (87,05-87,13)	co-regulator of the transcriptional activator <i>GATA1</i> , which regulates the expression of certain genes during erythroid and megakaryocytic differentiation <sup>44</sup>	<i>GATA-1</i> knockout mice are not viable. Mice with selective loss of <i>GATA-1</i> expression in megakaryocytes have thrombopenia.	no data available	Freson et al. demonstrated a disturbed interaction between mutants of <i>GATA1</i> and <i>FOG1</i> in patients with macrothrombocytopenia and dyserythropoiesis. <sup>44</sup>	neonatal thrombocytopenia in patient 2 and 3  unexplained macrocytosis in patient 3
<i>CBFA2T3</i> (MIM 603870) (87,47-87,57)	regulates the proliferation and the differentiation of erythroid progenitors	no data available	no data available	-	unexplained macrocytosis and neonatal thrombocytopenia in patient 3
<i>CDT1</i> (87,39-87,40)	transcriptional regulator (initiation) <sup>45</sup>	no data available	no data available	-	?



indicated. Interestingly, the deletion interval of patients 2 and 3 with transient thrombocytopenia comprises the gene *ZFPM1* (*FOG1*) (MIM 601950) which is involved in the regulation of the expression of genes during erythroid and megakaryocytic differentiation. Based on the available data, we were not able to recognize a candidate gene for the congenital heart defect in patient 3.

In conclusion, we hypothesize that *ANKRD11* and *ZNF778* are strong candidate genes for the observed core phenotype of this novel 16q24.3 microdeletion syndrome comprising autism spectrum disorder, variable cognitive impairment, facial dysmorphism and brain abnormalities, although a role of one or more of the other genes in the deleted regions of the individual patients is not excluded. Moreover, the involvement of *ANKRD11* and *ZNF778* warrants further studies searching for *ANKRD11* and/or *ZNF778* mutations in patients with a similar phenotype, but without 16q24 abnormalities by molecular karyotyping. Follow up of known patients might give answers to the possible occurrence of other phenotypic features. Moreover, the identification of new cases with overlapping deletions is needed to extend phenotypic knowledge and to confirm or even further narrow down the critical region of involved genes.

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### Resources

Database of Genomic Variants <http://projects.tcag.ca/variation/>; Decipher <sup>v4.1</sup> Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources <https://decipher.sanger.ac.uk/application/>; European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations (ECARUCA) <http://ecaruca.net>; Ensembl genome browser <http://www.ensembl.org>; Unigene <http://www.ncbi.nlm.nih.gov/UniGene>; USC Genome Browser (Assembly May 2004) <http://genome.ucsc.edu>; UniProt KB, Assembly July 2008 <http://www.uniprot.org>

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# 3.2.2

## Chromosome 1p21.3 microdeletions comprising *DPYD* and *MIR137* are associated with intellectual disability

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## Abstract

MicroRNAs (miRNAs) are non-coding gene transcripts involved in post-transcriptional regulation of genes. Recent studies identified miRNAs as important regulators of learning and memory in model organisms. So far, no mutations in specific miRNA genes have been associated with impaired cognitive functions.

In three sibs and two unrelated patients with intellectual disability (ID), we detected overlapping 1p21.3 deletions by genome-wide array analysis. The shortest region of overlap included *dihydropyrimidine dehydrogenase (DPYD)* and *microRNA 137 (MIR137)*. *DPYD* is involved in autosomal recessive dihydropyrimidine dehydrogenase deficiency. Hemizygous *DPYD* deletions were previously suggested to contribute to a phenotype with autism spectrum disorder and speech delay. Interestingly, the mature microRNA transcript microRNA-137 (miR-137) was recently shown to be involved in modulating neurogenesis in adult murine neuronal stem cells. Therefore, we investigated the possible involvement of *MIR137* in the 1p21.3-deletion phenotype. Our patients displayed a significantly decreased expression of both precursor and mature miR-137 levels, as well as significantly increased expression of the validated downstream targets *microphthalmia-associated transcription factor (MITF)* and *Enhancer of Zeste, Drosophila, Homologue 2 (EZH2)*, and the newly identified target *Kruppel-like factor 4 (KLF4)*. We also demonstrated significant enrichment of miR-137 at the synapses of cortical and hippocampal neurons, suggesting a role of miR-137 in regulating local synaptic protein synthesis machinery. This study showed that dosage effects of *MIR137* are associated with 1p21.3 microdeletions and may therefore contribute to the ID phenotype in patients with deletions harbouring this miRNA. A local effect at the synapse might be responsible.

## Introduction

microRNAs (miRNAs) are small RNAs that are highly conserved and involved in several biological processes like developmental timing, cell proliferation, apoptosis, metabolism, cell differentiation, and morphogenesis.<sup>1</sup> They regulate gene expression by binding to the 3'-untranslated region (UTR) of target mRNAs and inhibiting protein synthesis or causing mRNA degradation.<sup>2,3</sup> Each miRNA can act on many mRNAs, and conversely, individual mRNAs can be targeted by a number of miRNAs. Recent estimate puts the number of human miRNAs at 1100 or more, influencing the expression of as many as a two-third of all genes.<sup>4,5</sup> A few miRNAs, for example miR-124, miR-134 and miR-338 are specifically expressed in brain, suggesting unique regulatory roles in neuronal development and function.<sup>5,6</sup> The large number of miRNAs imposes a formidable challenge for studying miRNAs, their target mRNAs, and their functions in learning and memory, brain development and brain plasticity.<sup>7</sup> Multiple lines of evidence suggest that altered neuronal plasticity and morphology as seen in neurodevelopmental disorders may result from disruption of a common post-translational process that is under tight regulation by miRNAs.<sup>5,7</sup> Several intellectual disability (ID) syndromes, such as Fragile X syndrome, Rett syndrome, and Down syndrome, have been associated to miRNA pathways.<sup>8-12</sup> Little is known, however, about the pattern of expression of these small non-coding RNA molecules in the brains of normally developing individuals or patients with disorders of the nervous system. Moreover, no previous reports linked the disruption of specific microRNAs to ID.

While interstitial deletions of the short arm of chromosome 1 are rarely reported, a recently published study identified hemizygous microdeletions restricted to the chromosomal region 1p21.3.<sup>13</sup> The individuals described had autism spectrum disorder and severe speech delay. The authors of this study suggested that hemizygous deletions of the *DPYD* locus are contributing to these phenotypes. Though, absence of reports of abnormal cognitive functioning in the many heterozygous carriers of *DPYD* loss-of-function mutations opposes against an exclusive role for *DPYD* in the observed cognitive impairment.<sup>14</sup> *DPYD* is involved in autosomal recessive dihydropyrimidine dehydrogenase (DPD) deficiency. The phenotype is variable, but frequently comprises developmental delay and convulsions.<sup>15</sup>

In the present study, we have collected five individuals with ID who have overlapping 1p21.3 microdeletions that included *DPYD* but also the miRNA gene *microRNA 137 (MIR137)*. Expression studies revealed reduced levels of precursor and mature microRNA-137 (miR-137) and increased levels of downstream targets of miR-137 in the patients compared to controls. Our studies indicate that alteration in miR-137 expression levels is a likely explanation for their ID phenotype. To our knowledge,

this is the first study that demonstrates that a reduced copy number of a specific miRNA might be associated with ID.

Patients and Methods

Patients

Patients 1,2 and 4 were ascertained by genome-wide array analysis during a pending cohort study in patients with unexplained ID at the department of Human Genetics in Nijmegen, The Netherlands. Patient 5 was ascertained by genome-wide array analysis during routine diagnostic evaluation of her ID at the departments of clinical genetics and neuropsychiatrics in Lille, France. Patients 1-3 were siblings. From patient 3 we obtained a blood sample for genetic testing and concise information on her developmental level, but it was not possible to perform a clinical examination. Chromosomal analysis in the parents of the three affected sibs was not possible, because they were deceased. They were known with low cognitive abilities and had not been able to take care for their children, who were placed in foster homes. Patients 4 and 5 were single unrelated patients. Fragile X syndrome and Prader Willi syndrome were excluded in patients 1 and 2 from family 1 and in patients 4 and 5.

SNP array analysis

DNA was obtained from peripheral blood leucocytes and isolated according to standard procedures. In patients 1-4, as well as in the parents of patient 4, genome-wide Single Nucleotide Polymorphism (SNP) array analysis was performed by the Affymetrix 250k SNP array platform according to the standard Affymetrix GeneChip protocol (Affymetrix, Inc., Santa Clara, CA, USA). In patient 5 and her parents genome-wide array analysis was performed by the Agilent 44k array platform. Copy number estimates were determined using the updated version 2.0 of the CNAG (Copy Number Analyzer for Affymetrix GeneChip mapping) software package.<sup>16</sup> The normalized ratios were subsequently analyzed for genomic imbalances by a standard Hidden Marker Model (HMM). CNVs were mapped according to the USCS genome browser build March 2006. To confirm the deletions detected by 250k SNP array analysis, quantitative Polymerase Chain Reaction (qPCR) analysis on DNA extracted from blood lymphocytes was performed.

Metabolic tests

In patients 1 and 2 (siblings) and in patient 4 measurements of serum and urine levels of purines and pyrimidines were performed to rule out DPD deficiency.

RNA isolation

1 to 5 micrograms of total RNA were isolated from cell suspensions of approximately 1x10<sup>6</sup> EBV-transformed B cells from individual cell lines using the NucleoSpin RNA II RNA isolation kit from Machery-Nagel (Düren, Germany). The concentration and quantity of RNA were measured by UV absorbance at 260 and 280 nm (A260/280 ratio) and checked by gel electrophoresis.

Quantitative reverse transcription-PCR (qRT-PCR) of precursor and mature miRNA-137 and its downstream targets

qRT-PCR quantification of precursor and mature miR-137 levels and its validated and newly identified downstream targets was performed on RNA from lymphoblastoid cell lines (LCL) of patients and controls, as well as on RNA from several human brain regions and mouse cortical synaptosomal preparations, according to previously described protocols.<sup>17, 18</sup> Precursor miR-137 primers were designed to anneal within the hairpin sequence of the miRNA-137 precursors. Precursor miRNA-137 cDNA was synthesized from total RNA by using gene-specific miR-137 reverse PCR primers. The forward primers for qPCR analyses for mature miR-137 were designed using the entire mature miRNA sequence (primer sequences are shown in Table 1).

Table 1 Primers used for qRT-PCR of precursor and mature miR-137

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
U6 snRNA	CTCGCTTCGGCAGCAC	AACGCTTCACGAATTTGCGT
rno-miR-137	GTGACGGGTATTCTTGGGT	GACTACGCGTATTCTTAAGCAA

Mature miR-137 cDNA was synthesized from total RNA (1μg) using the NCode miRNA First Strand cDNA synthesis kit (Invitrogen) according to the manufacturer's protocol. Using first-strand cDNA as a template, triplicate qPCR reactions were performed from samples derived from 3 different experiments for mature miRNA using miR-137-specific forward primers and the NCode Universal qPCR reverse primer. Minus template and minus forward primer controls were included to ensure lack of signal in the assay background. U6 snRNA levels were used as a loading control. All Ct values used for analyses were averaged from three to six replicates and those with high standard deviation (>1) were not included in the analyses. Melting curve analysis was performed to test for the specificity and quality of the qPCR amplifications. Relative expression was calculated using the comparative CT method<sup>18</sup> normalized to the expression of U6 snRNA. To assess the levels of



miR-137 downstream target mRNAs, Two  $\mu\text{g}$  of DNase-treated, total RNA from each sample was used for cDNA synthesis, using the RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas Inc., USA). Prior to qPCR analysis, each cDNA sample was diluted 1:10 with MilliQ water. qPCR was performed according to previously described protocols<sup>17, 18</sup>, using standard cycling conditions, and performing a melting protocol to control for product specificity. The Maxima™ SYBR Green qPCR Master Mix Kit (Fermentas Inc., USA) was used in the qPCR reactions. Primers were designed using NCBI Primer-Blast ([www.ncbi.nlm.nih.gov/tools/primer-blast/](http://www.ncbi.nlm.nih.gov/tools/primer-blast/)) and synthesized at Biolegio BV (The Netherlands). Primer sequences for *MITF*, *EZH2*, and *KLF4* are available upon request. The relative levels of the transcripts of the downstream targets were normalized to *beta-glucuronidase (GUSB)* and *beta-actin (ACTB)* mRNAs to provide an internal control for reverse transcription. These messages were used as reference genes, because of their stable expression in lymphocytes.<sup>19</sup> All other human RNAs investigated were purchased from Stratagene (La Jolla, CA, USA).

### Synaptosomal preparation

Synaptosomes were isolated based on the method of Gray and Whittaker (1962).<sup>20</sup> All the procedures were performed at 4 °C, and under RNase-free conditions. Briefly, mouse cortical tissue was homogenated using a Dounce Homogenizer (B. Braun Biotech International) in 5mM HEPES buffer containing 0.32 M sucrose, protease inhibitor cocktail (Roche) and 1U/mL RNase inhibitor (Fermentas). The homogenate was centrifuged at 1000x g for 10 min, obtaining supernatant S1 and pellet P1. Pellet P1 was resuspended with homogenization buffer and centrifuged at 1000x g for 10 min, obtaining supernatant S2 and pellet P2 (containing the nuclear fraction). Supernatants S1 and S2 were pooled together and centrifuged at 20,000x g for 10 min, obtaining supernatant S3 (containing the cytoplasmic fraction) and pellet P3. Pellet P3 was resuspended in homogenization buffer, loaded on a discontinuous sucrose density gradient (0.85 M and 1.2 M sucrose layers), and centrifuged at 30,000x g for 2h. The interface between the two sucrose layers was collected and centrifuged at 25,000x g for 30 min, obtaining a pellet containing the synaptosomal fraction. The purity of the synaptosomal fraction was determined by Western blot assay using the synaptic marker (PSD95, 1:5000; NeuroMab) and markers known not to be enriched at the synapse (tubulin, 1:500; in-house antibody). GADPH (1:1000; Cell Signaling) was used as a housekeeping marker. The nuclear, cytoplasmic and synaptosomal fractions were kept at -80 °C until further analysis. RNA from nuclear, cytoplasmic and synaptosomal fractions was isolated with TRIzol® Reagent (Invitrogen), according to the manufacturers protocol. The procedure was modified for small amounts of tissue by using 800  $\mu\text{L}$  of TRIzol® Reagent and adding 1  $\mu\text{L}$  of glycogen (Fermentas). RNA concentration

and quality was determined with a Nanodrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific) and 1% agarose gel electrophoresis, respectively. The samples were kept at -80 °C until further analysis.

### miRNA computational predictions

To assess the potential regulatory impact of miR-137 at the synapse, we searched the TargetScan miRNA target database for conserved, putative rodent targets of miR-137.<sup>21, 22</sup>

### Neuronal cell cultures and transfections

Primary cultures of cortical neurons were prepared from embryonic day 18 rats as described<sup>23</sup> and maintained in a neurobasal medium supplemented with B27 (Invitrogen, Carlsbad, CA, USA) and 2 mmol/L glutamine. For miR-137 expression experiments, the miRNASelect™ pEGP-mmu-mir-137 Expression Vector (Cell Biolabs, Inc., San Diego, CA) constructs expressing mouse miR-137 precursor and GFP, or the negative control pEGP-null vector (expressing GFP) were purchased. Locked nucleic acid miR inhibitor, anti-miR-137, as well as nontargeting control anti-miR-NT were obtained from Exiqon (Denmark). Small RNAs and Constructs were introduced into primary neurons using Lipofectamine 2000 (Invitrogen).

### Statistical Analysis

Student's t test was used to determine significant differences in transcript levels between the patients and the controls.

One-way ANOVA was used to analyze significant differences among multiple groups;  $p < 0.05$  was considered significant.

## Results

### Clinical results

A summary of the clinical features is shown in Table 2. Photographs of patients 1, 2, 4 and 5 are shown in Figure 1. Though the patients have no distinct dysmorphic facial features, they showed similarities in facial appearance, including long ears, deeply set eyes, broad nasal tip and thick lower lip. The level of ID ranged from borderline-mild (patient 1) to mild-moderate (patient 2 and 3). Remarkably, patients 1, 2 and 4 showed a similar discrepancy between verbal and performal IQ with a relatively low score on verbal capacities. Such a discrepancy was not reported in patient 5. Patient 2 of family 1 and patient 4 also showed similar speech deficits including poor intelligibility and specific pronunciation deficits (in patients 1, 3 and 5 speech tests were not performed). Similar behaviour characteristics included a

remarkable shy and friendly behaviour, (tendency) to overeating and features of autism spectrum disorder (ASD). Patients 1, 2, 4 and 5 were obese or had a tendency to obesity. Patients 1, 2 and 4 had a-specific ocular problems.

Serum and urine measurements of purine and pyrimidine levels in patients 1, 2 and 4 revealed no abnormalities indicative for DPD deficiency.

### Genome-wide array analysis

Figure 2 provides a schematic representation of the overlapping deletions in 1p21.3 in our patients and in the patients reported by Carter and others.<sup>13</sup> In patients 1-3 an identical interstitial 1.75 Mb loss was identified (97,50-99,25 Mb, according to Hg 18 UCSC genome browser): 46, XY/XX.arr snp 1p21.3 (SNP\_A-2211294→SNP\_A-2131140)x1.

**Table 2** Clinical and molecular features of overlapping 1p21.3 microdeletions in patients 1-5

Patient	1*	2*	3*	4	5	Overlapping region
<b>Size of deletion</b>	1.75 Mb	1.75 Mb	1.75 Mb	1.41 Mb	2.45 Mb	1.22 Mb
<b>Mb positions (Hg 18)</b>	97.50-99.25	97.50-99.25	97.50-99.25	97.32-98.73	96.27-98.72	97.50-98.72
<b>Involved genes</b>	<i>DPYD, SNX7, LPPR5, MIR137</i>	<i>DPYD, SNX7, LPPR5, MIR137</i>	<i>DPYD, SNX7, LPPR5, MIR137</i>	<i>DPYD, MIR137</i>	<i>PTBP2, DPYD, MIR137</i>	<i>DPYD, MIR137</i>
<b>Gender</b>	male	male	Female	Male	female	
<b>Age at clinical examination</b>	42 years	38 years	-	33 years	18 years	
						<b>Total</b>
<b>Intellectual disability</b>	borderline-mild (TIQ 73, verbal IQ 65, performat IQ 90)	mild-moderate (TIQ 52, verbal IQ < performat IQ (70)	mild-moderate	mild (TIQ 62, verbal IQ 59, performat IQ 71)	mild (TIQ 66)	5/5
<b>Behaviour characteristics</b>			unknown			
Features of ASD	+	+		+	-	3/4
(Tendency) to overeating	+	+		+	-	3/4
Self mutilation	-	+		-	-	1/4
Aggressive outbursts	-	+		-	+	2/4
Remarkable shy and friendly	+	+		+	+	4/4
<b>Specific speech deficits</b>	NT	+	NT	+	NT	2/2
<b>Weight</b>	90 <sup>th</sup> centile	>98 <sup>th</sup> centile	unknown	98 <sup>th</sup> centile	>98 <sup>th</sup> centile	4/4 (tendency) to overweight
<b>Facial characteristics</b>			unknown			
Deeply set eyes	+	-		+	-	2/4
Full cheeks	-	-		+	+	2/4
Micrognathia	-	-		+	-	1/4
Broad nasal tip	+	+		+	-	3/4
Long ears	+	+		+	+	4/4
Macrostomia	-	+		-	-	1/4
Thick lower lip	+	+		+	+	4/4
<b>Ocular problems</b>	myopia	astigmatism	unknown	astigmatism, myopia	-	3/4

\*patients 1-3 are sibs. ASD= autism spectrum disorder. NT= not tested. TIQ: total intelligence quotient

**Figure 1** Facial appearance of patients 1, 2, 4 and 5.

Figures **A-B** show patient 1 at age 42 years, figures **C-D** patient 2 at age 38 years, figures **E-F** patient 4 at age 34 years and figures **G-H** patient 5 at age 18 years. Note the minor facial dysmorphic features, including long ears, deeply set eyes, full tip of the nose and thick lower lip.



In patient 4 an interstitial loss of 1.41 Mb was identified (97,32-98,73 Mb, according to Hg 18 UCSC genome browser): 46,XY, 1p21.3 (SNP\_A-1881473→SNP\_A-1789641) x1. In patient 5 an interstitial loss of 2.45 Mb was identified (96,27-98,72 Mb, according to Hg 18 UCSC genome browser): 46,XX, 1p21.3 (probe A\_14\_P100140 → probe A\_14\_P113179). The deletion of patients 1-3 comprised 3 annotated Refseq genes, including *DPYD*, *SNX7* and *LPPR5*, and the microRNA gene *MIR137*. The deletion of patient 4 comprised the annotated gene *DPYD* and *MIR137* and the deletion of patient 5 comprised the annotated genes *PTBP2* and *DPYD* and *MIR137*. The shortest region of overlap is 1.22 Mb in size and included *DPYD* and *MIR137*. Genome-wide array analysis in the parents of patient 4 and 5 showed that the deletions in these patients had occurred *de novo*. DNA samples from the parents of patients 1-3 were not available for segregation analysis, because they were deceased.

**Figure 2** Schematic representation of the overlapping deleted regions in chromosome 1p21.3 identified by genome-wide array analysis

The box indicates the chromosomal location that is schematically depicted below. The relative positions of the deleted regions in patients 1-3, 4 and 5 are indicated by black bars. The deletions in the patients reported by Carter and others<sup>13</sup> are indicated in gray. The relative positions of the genes *PTBP2*, *DPYD*, *SNX7*, *LPPR5* and *MIR137* are indicated. The deletion of patient 5 extends distally beyond the figure. SRO: shortest region of overlap (in the present patients).

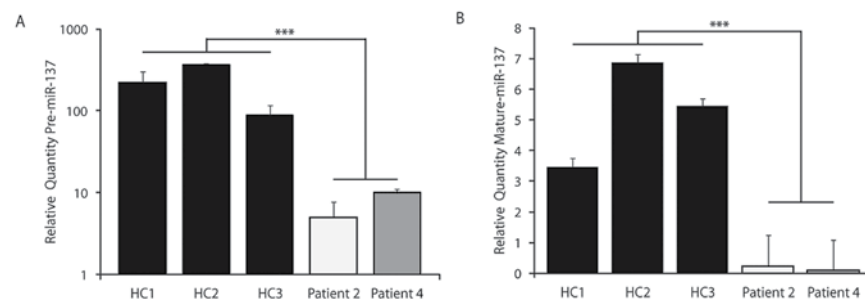
### Differential miR-137 expression in LCL from the patients

To study the effect of loss of one copy of *MIR137*, we performed qRT-PCR on precursor and mature miR-137 in patient 2 (one of the siblings) and patient 4. LCL from patient 5 were not available. As controls we used RNA extracted from the LCL of 3 healthy male controls. Decreased precursor and mature miR-137 levels were found in the LCL from patient 2 as well as the LCL from patient 4, as compared to healthy controls and normalized to snoRNA U6 (one-way ANOVA,  $p < 0.001$ ) (Figure 3). The results demonstrate a great degree of reduction of miR-137 levels in the patients.

### Increased levels of MITF and EZH2 in patient cell lines

While the major effect of miRNA's has been proven to be on the translation of target mRNAs, recent studies indicate that miRNAs also have a specific impact on the mRNA levels.<sup>3,24</sup> We therefore investigated whether reduced levels of miR-137 in our patients corresponded to an increased level of the previously validated miR-137 targets *EZH2*<sup>25</sup>, and *MITF*.<sup>26</sup> To assess miR-137-mediated modulation of *EZH2* and

**Figure 3** Precursor and mature miR-137 levels are decreased in the patients. The quantitative RT-PCR assay was used to assess levels of precursor and mature miR-137 isolated from either the LCL of patients 2 and 4 and 3 healthy controls (HC). Levels of precursor (A) and mature (B) miR-137 levels are expressed relative to U6 snRNA which was used as an internal control.



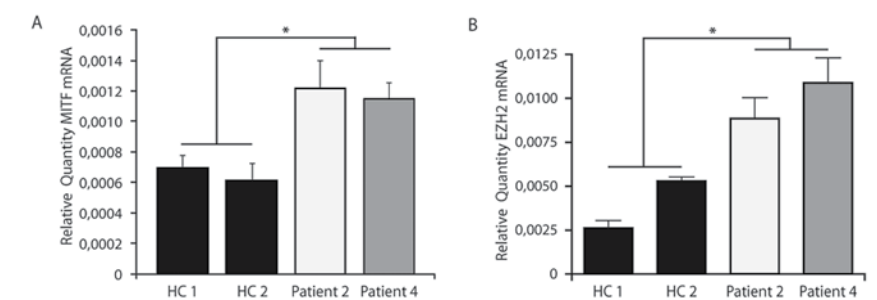
Error bars represent the Standard Error of the Mean (SEM) for triplicate PCR reactions from n=3 independent experiments; one-way ANOVA, \*\*\*  $p < 0.0001$ .

*MITF* mRNA levels, we monitored transcript levels in LCL of our patients and healthy controls by qRT-PCR. This investigation revealed significantly increased *MITF* and *EZH2* levels in the patients as compared to two healthy controls (Figure 4).

### miR-137 is enriched in the hippocampus and cortical brain regions, and highly abundant at the synaptodendritic compartments of neurons

Many miRNAs have been isolated from mammalian embryonic neurons and mature brains and some of them are neuronal-specific.<sup>27</sup> Although miR-137 is specifically expressed in neuronal tissue<sup>28</sup>, little is known about its abundance and function during neuronal maturation. To analyze the miR-137 level expression patterns in embryonic and mature brain, qRT-PCR was performed on total RNA isolated from fetal and adult (post-mortem) human brain. As shown in Figure 5A, miR-137 levels are approximately 100 times higher in the adult as compared to embryonic brain, suggesting that the expression of miR-137 is increasing during brain development and during neuronal differentiation indicating that it might have an important role in human brain development and functioning.

**Figure 4** Validated miR-137 targets *MITF* and *EZH2* mRNA levels are increased in the patients. The qRT-PCR assay was used to assess mRNA levels of *MITF* (A) and *EZH2* (B) isolated from either the LCL of patients 2 and 4, and 2 healthy controls. mRNA levels are expressed relative to  $\beta$ -actin and *GusB* which were used as an internal controls.



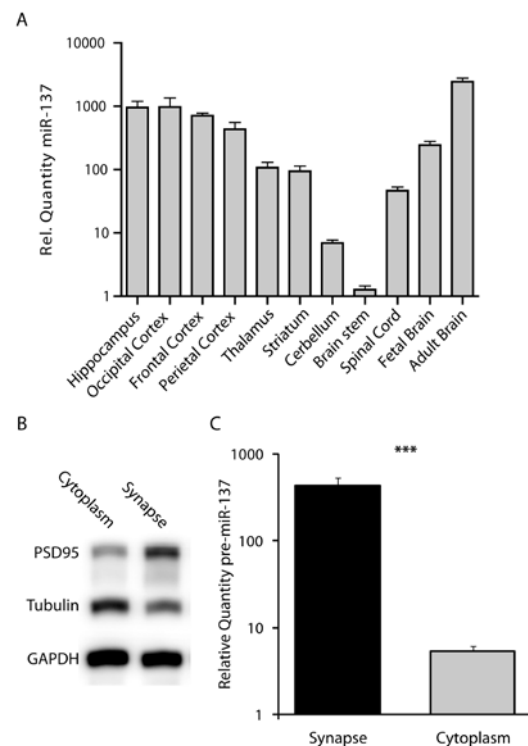
Error bars represent the SEM for triplicate PCR reactions from n=3 independent experiments; one-way ANOVA, \*  $p < 0.05$ .

Using commercially available RNA isolated from a subset of human brain compartments, we showed that miR-137 is most abundant in the hippocampus. Similarly, high miR-137 levels can be detected in the occipital, frontal, as well as the parietal cortical regions, while the lowest miR-137 levels were detected in the brain stem and cerebellum (Figure 5A).

Recent findings indicate that miRNAs can be enriched in synaptic compartments of neurons.<sup>29</sup> This notion suggests that miRNAs may be ideally positioned to quickly regulate local translation in response to synaptic activity.<sup>30</sup> A recent study revealed that the neuron-enriched miR-137 has a prominent role in the synapse maturation and morphogenesis of young neurons.<sup>31</sup> We therefore hypothesized that miRNA-137 might reside near the synapse, where it could act as local regulator of the translation of synaptically enriched target mRNAs. Recent studies employed biochemically isolated synaptosomes from young rat brains to demonstrate that these small vesicular structures preserve components of local protein synthesis, including polyribosomes, mRNAs and regulatory RNAs, including microRNAs.<sup>32, 33</sup> We used differential fractionation to isolate mouse synaptosomal compartments, and the preparations were assessed by Western blotting using PSD95, GAPDH, and tubulin antibodies (Figure 5B), as well as by real-time RT-PCR measurements of *CAMK2a*,



**Figure 5** miR-137 is overexpressed in the hippocampus and cortical brain regions, and enriched at synaptic compartments. **(A)** Differential brain regional distribution of miR-137. The qRT-PCR miRNA assay was used to quantify precursor mir-137 across different human brain regions. **(B)** Differential fractionation was used to isolate mouse synaptosomal compartments, and the preparations were assessed by western blotting using PSD95, GAPDH and tubulin antibodies. **(C)** qRT-PCR based quantification of precursor mir-137 isolated from the synaptosomal and the cytosolic fractions of mouse brain. Levels of precursor mir-137 are expressed relative to U6 which was used as an internal control.



Error bars represent the SEM for triplicate PCR reactions from n=3 independent experiments; t-test \*\*\* p<0.0001.

to verify that these components were enriched relative to total forebrain homogenate. This experiment revealed significant enrichment of miR-137 in RNA preparations from mouse synaptosomes compared to the cytosolic fractions, as assessed by qRT-PCR (Figure 5C). The outcome of this experiment suggests that miR-137 may have a local role in translation-dependent synaptic morphology.

### Regulation of neuronal levels of KLF4 mRNA by miR-137

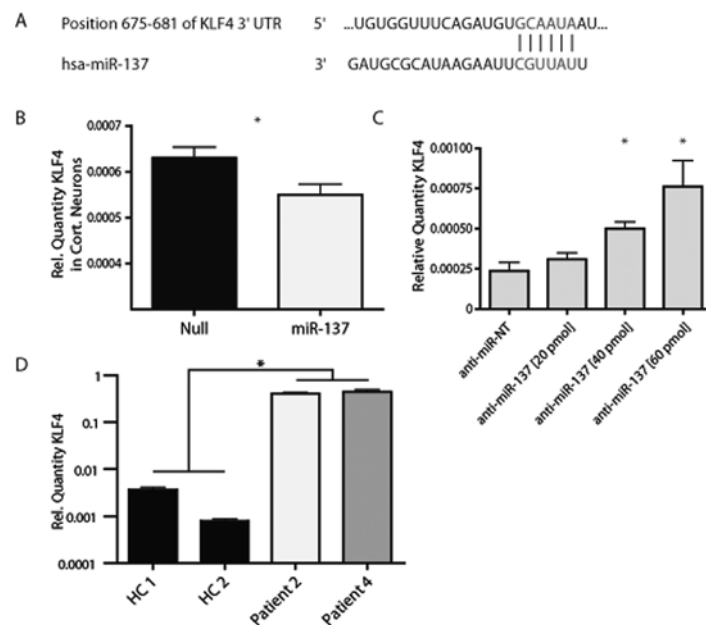
To further assess the potential regulatory impact of miR-137, we searched the TargetScan database for additional mRNAs that contained a putative miR-137 binding site.<sup>34</sup> This revealed a large number putative miR-137 target mRNAs (data not shown). Among these mRNAs are three members of the kruppel-like factor genes - *KLF4*, *KLF11*, and *KLF12*- which contained highly conserved binding sites for miR-137 in their 3'UTRs. Kruppel-like factor proteins (KLFs) are a family of transcriptional repressors associated with axon growth in central nervous system (CNS) neurons<sup>35</sup>, and previous studies identified coordinated activities of different KLFs to enhance the regenerative capacity of CNS neurons. The predicted miR-137 target site (MTS) in *KLF4* 3'UTR (Figure 6A) had a high aggregate P<sub>CT</sub> score with the cognate miR (0.77), suggesting a conserved targeting of the MTS for miR-137.<sup>36</sup> To explore whether miR-137 regulates *Klf4* mRNA levels in neurons, we monitored *Klf4* mRNA levels after transfecting rat cortical neurons (DIV 10) with a miR-137 precursor vector. miR-137 transfection with an expression vector resulted in a 1000-fold increase in mature miR-137 levels compared with the endogenous miR-137 levels in null vector-transfected cortical neurons (not shown). *Klf4* mRNA levels decreased 20% in miR-137 overexpressing cortical neurons when compared with the null-vector transfected neurons (Figure 6B). Conversely, transfection of neurons with a locked nucleic acid inhibitor of miR-137 resulted in a three-fold increase in *Klf4* mRNA as early as 72 hrs after transfection (Figure 6C), when compared with the non-targeting anti-miR control.

The results from the miR-137 overexpression or inhibition experiments in rat cortical neurons prompted us to investigate *KLF4* levels in LCL from our patients. This revealed significantly increased *KLF4* mRNA levels in the patients as compared to the healthy controls (Figure 6D).

## Discussion

We present evidence for likely involvement of *MIR137* in the ID phenotype of patients with 1p21.3 microdeletions. The shortest region of overlap included *DPYD* and *MIR137*. *DPYD* is involved in autosomal recessive DPD deficiency, which is characterized by developmental delay and convulsions.<sup>15</sup> Several arguments

**Figure 6** miR-137 target *KLF4*. (A) Putative miR-137 binding site in the rat *Klf4* 3' UTR. The 6 nt seed sequence is marked by vertical lines. (B) miR-137 overexpression reduces *Klf4* levels in rat primary cortical neurons. (C) Quantification of *Klf4* mRNA levels in rat cortical neurons transfected with anti-miR-137, or non-targeting short oligonucleotides (anti-miR-NT), as determined by qRT-PCR 72 hrs after oligonucleotide transfection. (D) *KLF4* mRNA levels are increased in patients 2 and 4. mRNA levels are expressed relative to  $\beta$ -actin which was used as an internal control.



Error bars represent the SEM for triplicate PCR reactions from  $n=3$  independent experiments; one-way ANOVA, \*  $p < 0.05$ .

oppose an (exclusively) causative role of deletion of *DPYD* in the ID phenotype of the presented patients. First, none of the present patients in whom metabolic tests were performed (patients 1, 2 and 4) showed the typical thymine-uraciluria associated with DPD deficiency. Second, although heterozygous mutations in *DPYD*, including loss-of-function mutations, occur frequently and contribute to toxicity for the anti-cancer drug 5-fluorouracil (5-FU)<sup>14</sup>, ID has not been reported in

heterozygous carriers. In a recent report, four patients from three families with overlapping microdeletions in the same region were described.<sup>13</sup> It was suggested that the associated phenotype, consisting of speech delay and autism, was due to hemizygous deletion of *DPYD*. However, one of the deletions in this report also comprised *MIR137* and the identical deletion reported in two siblings was located only 40 kb proximally from *MIR137* and might affect expression of *MIR137*. Finally, the fourth patient had an intragenic *DPYD* deletion which was inherited from a healthy mother and thus likely representing an inherited variant similar to many heterozygous carriers that are known. It is likely that one of the parents of sibs 1-3 from the present report also had the deletion, but both parents had impaired cognitive abilities and were not able to raise their children who, consequently, had been placed in foster care. Therefore, we conclude that at least for the patients with a heterozygous deletion encompassing *MIR137*, the phenotype is more likely associated with haploinsufficiency of *MIR137*, although a role for *DPYD* is not fully excluded. Indeed, previous reports of miR-137 studies showed that its expression is epigenetically regulated by *Methyl-CpG-binding protein 2 (MeCP2)* during different aspects of neurogenesis in mice.<sup>25,31</sup> Therefore, *MIR137* represents a good candidate for the observed ID phenotype in our patients.

There is one other example of the involvement of microRNA mutations in a human genetic disorder: mutations in *MIR96* cause autosomal dominant hearing impairment.<sup>37</sup> In addition some reports have shown that disruption of the recognition sites in target genes for specific miRNAs might be associated with a genetic trait in humans. In this regard, some evidence was provided that a mutation in the binding domain for miR-189 in the *SLITRK1* is associated with Tourette syndrome and other neuropsychiatric features.<sup>38</sup> Other studies have highlighted the importance of miRNA pathways in neuronal processes. A recent study<sup>39</sup> showed increased cognition of adult *Dicer1* mutant mice, lacking miRNAs (such as miR-137) in mature neurons in adult brain compared to controls, thereby providing evidence for a role of miRNAs as key players in learning and memory processes of mammals. In addition, recent findings indicate that miRNAs may be involved in modulating short-term synaptic plasticity.<sup>40</sup> Furthermore, synaptic activity can influence miRNA expression: recent reports indicated that miRNA levels are altered in hippocampal neurons that are induced to display long-term depression (LTD) or long-term potentiation (LTP).<sup>41, 42</sup> This finding suggests that different levels or variation in miRNA expression levels will affect translation of synaptic proteins and consequently synaptic plasticity. Expression studies of precursor and mature miR-137 in our patients and controls demonstrated significantly reduced expression levels in the patients. We performed these studies in LCL of the patients. A number of previous investigations assessed the relevance of using LCL as probes to study the genetics of neurodevelopmental disorders.<sup>43, 44</sup> Baron and others<sup>43</sup> assessed the feasibility of

using LCL for genome-wide expression profiling and showed the usefulness of this tool for identifying genes associated with neurodevelopmental disorders. As shown in Figure 3, the expression of miR-137 is even more than 2-fold reduced. This might be explained by the short half life of miR-137 in mammalian cells<sup>45</sup> in combination with an elevated synthesis of proteins involved in RNA turnover processes which are targeted by miR-137 (caused by a reduced inhibition of these proteins due to a reduced level of miR-137). This could further accelerate RNA turnover in the patients, which would result in lower miR-137 levels.

Our studies in mice also suggested an enrichment of miR-137 in RNA preparations of mouse synaptosomes, corresponding with the results of a recent study that revealed a significant role of the neuron-enriched miR-137 in the synapse maturation and morphogenesis of young neurons.<sup>31</sup> This suggests that miR-137 may have a local role in translation-dependent synaptic morphology and that defects in miR-137 could interfere with proper synaptic signal transduction, thereby affecting cognitive brain function. We also showed a significantly increased expression of the previously validated downstream targets *MITF* and *EZH2* and the newly identified downstream target *KLF4*. *MITF* encodes a transcription factor that is involved in the regulation of differentiation and development of melanocytes in the retinal pigment epithelium. Heterozygous loss-of-function mutations in *MITF* are involved in Waardenburg syndrome type 2A [MIM 193510] and Tietz syndrome [MIM 193510]. As expected, our patients, who have an opposite dosage effect of *MITF*, did not show the classic features of these syndromes (hypopigmentation and deafness).

The downstream target *EZH2* is a histone H3 lysine 27 methyltransferase and a member of the Polycomb protein family. It functions as a transcriptional repressor. The protein regulates CpG methylation by direct interaction with DNA methyltransferases.<sup>46</sup> Histone methylation plays an important role in modulating chromatin structure and function. It was previously shown that the *EZH2* protein interacts with the *ATR-X* gene which is involved in alpha-thalassemia/mental retardation syndrome (ATR-X-syndrome) [MIM 301040].<sup>47</sup> Furthermore, several other genes that are associated with ID phenotypes, such as *MECP2*<sup>48</sup> and *EHMT1*<sup>49</sup> are involved in chromatin modulation. Therefore, an association between altered expression of the downstream target *EZH2* with the ID phenotype in our patients might very well be possible. In addition, the here newly identified downstream target *KLF4* might contribute to the ID phenotype as well, since it was shown to act as a transcriptional repressor of axon growth in central nervous system.<sup>35</sup> The reduced expression of miR-137 likely results in significantly altered expression of other putative miR-137 targets as well. Moreover, the identification of additional aberrations in *MIR137* in other patients with ID and normal genome-wide array results would provide further evidence for the role of *MIR137*. In our in house database, comprising the results of genome wide array analysis in more than 4,000 ID patients and controls, no

additional deletions restricted to 1p21.3 have been registered. In addition we screened a large cohort of approximately 1000 ID patients who had been previously tested negative for Fragile X and/or Prader Willi syndrome and had normal karyotypes, for *MIR137* aberrations. We selected this cohort of patients because no designated phenotypic features were present except ID and overweight. We have not found additional *MIR137* aberrations (results not shown), suggesting that disruption of *MIR137* would be at any rate a rare cause of ID.

To further unravel the underlying mechanism, additional expression studies of other known downstream targets that are expressed in the dendrites are warranted. Furthermore, future studies are needed to further determine the synaptic role of miR-137 as a regulator of local translation in dendrites which is known to be a pivotal mechanism of synaptic maturation and plasticity.

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We thank the participating patients and parents.

### Competing interests

No competing interest reported.

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# 3.2.3

## **Interpretation of clinical relevance of X-chromosome copy number variations identified in a large cohort of individuals with cognitive disorders and/or congenital anomalies**

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## Abstract

Genome-wide array studies are now routinely being used in the evaluation of patients with cognitive disorders (CD) and/or congenital anomalies (CA). Therefore, inevitably each clinician is confronted with the challenging task of the interpretation of copy number variations detected by genome-wide array platforms in a diagnostic setting. Clinical interpretation of autosomal copy number variations is already challenging, but assessment of the clinical relevance of copy number variations of the X-chromosome is even more complex. This study provides an overview of the X-Chromosome copy number variations that we have identified by genome-wide array analysis in a large cohort of 4,407 male and female patients. We have made an interpretation of the clinical relevance of each of these copy number variations based on well-defined criteria and previous reports in literature and databases. The prevalence of X-chromosome copy number variations in this cohort was 57/4,407 (~1.3%), of which 15 (0.3%) were interpreted as (likely) pathogenic.

## Introduction

Both intellectual disability (ID) and autism spectrum disorders (ASD) are relatively frequent in the population, with an estimated prevalence of 2-3% and 1% respectively.<sup>1,2</sup> The prevalence of both disorders is higher in males than in females.<sup>2,3,4</sup> ID and ASD are clinically and genetically heterogeneous. Genetic factors are expected to play a causal role in a substantial part of the cases.<sup>5-10</sup> A large proportion of patients with ID have ASD as well, suggesting that shared underlying genetic and biological defects may be involved.<sup>11</sup>

In the recent years genome wide array technologies including array comparative genomic hybridization (array-CGH) and single nucleotide polymorphism oligonucleotide arrays (SNP array) have become routine tools in the clinical evaluation of patients with ID and/or congenital anomalies (CA) and are increasingly used in the evaluation of other neuropsychiatric disorders as ASD, ADHD and schizophrenia as well. This has led to the identification of several causative submicroscopic chromosomal aberrations/copy number variations (CNVs), including X chromosomal aberrations, that are too small in size to be detected by routine cytogenetic and molecular cytogenetic techniques. Among previously reported genome-wide array studies in unselected cohorts of patients with ID, CA and/or dysmorphic features, the reported detection rate of X-chromosome CNVs (X-CNVs) varied from 0% to about 40%, but in the majority of the studies prevalence ranged from 0-5%. These figures are influenced by the low total number of patients in some studies, differences in patient selection, the use of different array platforms with variable probe coverage on the X-chromosome and different detection limits. Some studies only reported pathogenic X-CNVs.<sup>12-33</sup> Studies using X-chromosome specific array-CGH reported a higher detection rate of X-CNVs which is also partly explained by the selection of a cohort of predominantly male patients with the suspicion of an X-linked mode of inheritance.<sup>34-40</sup> Studying the X-chromosome aberrations detected by X chromosome specific arrays has led to the identification of novel X-linked ID genes, like *ZNF674* in non-syndromic X-linked ID.<sup>41</sup> Such studies have also identified deletions and duplications of chromosomal regions that contain known X-linked ID genes, with duplications of the Xq28 region encompassing the *MECP2* gene being the most frequently identified causative X-CNV.<sup>42</sup>

Genome-wide array studies in cohorts of patients with ASD revealed several associated CNVs in this group of patients as well, including X-CNVs. Clinical relevant CNVs are detected in about 5-8% of ASD patients.<sup>10,43</sup> Interestingly, several of these CNVs are present both in cohorts of patients with ID, and in cohorts of patients with ASD. In addition, such aberrations have also been identified in patients with other neuropsychiatric disorders like Attention Deficit Hyperactivity Disorder (ADHD) and schizophrenia. Well-known examples are 15q11q13 duplications,

22q11.21 deletions and 16p11.2 deletions and duplications.<sup>43</sup> The study of CNVs in ASD revealed a number of ASD related genes as well, such as *NRXN1*, *CNTN4*, *NLGN1* and *ASTN2*.<sup>44</sup> Most of these genes are associated with ID as well. This is another indication for involvement of similar underlying molecular and biological pathways in the etiology of variable cognitive disorders (CD), as ID/developmental delay (DD), ASD and other neuropsychiatric disorders.

Whole-genome scanning technologies have also identified a large number of submicroscopic CNVs that could not be directly associated with CD and/or CA. Furthermore, many CNVs, either on the autosomes or the X-chromosome, are also present in the general population, giving rise to difficulties in the interpretation of these CNVs.<sup>45-47</sup> X-CNVs form a special group, because dosage effects likely differ in males and females and causative CNVs in a male patient can be inherited from a healthy mother.

In total, various studies reported over 100 X-CNVs in control individuals, which are collected in databases<sup>37</sup> (Database of Genomic Variants: <http://projects.tcag.ca/variation>).

In the present study we provide an overview of X-CNVs identified by genome wide array analysis in a cohort of 4,407 individuals with various clinical presentations of CD, and/or CA, including an interpretation of the clinical relevance. Our aim was to provide a resource for clinicians and laboratory specialist for the interpretation of X-CNVs that are picked up by genome wide array platforms in a routine diagnostic setting.

## Patient Data

### Ascertainment of patients

Our data are derived from genome wide copy number profiling performed among a cohort of 4,407 individuals. All individuals had been referred between January 2003 and August 2010 to our diagnostic center for the evaluation of cognitive disorders (CD) -including unexplained ID/DD, neuropsychiatric disorders- and/or CA. Neuropsychiatric disorders included mainly autism spectrum disorders (ASD) and Attention Deficit Hyperactivity Disorder (ADHD). In the majority the indication for genome-wide array analysis was DD/ID (with or without other neuropsychiatric disorders and/or CA). In a minority of the patients genome-wide array analysis was performed because of CA or behavior problems *without* presence of DD/ID. Two third of the total cohort had an age between 1 and 18 years. The male to female ratio was 1.2:1.

## Methods

### Genome-wide copy number profiling

Genome-wide copy number profiling was either performed by the Agilent 32 k BAC array (669 patients) or the Affymetrix 250k SNP array (3,738 patients) analysis platform. Since 2009, genome-wide 250k SNP array analysis has replaced routine cytogenetic chromosome studies as the first line diagnostic test for patients with CD and/or CA in our laboratory.

Genome-wide 32k BAC array analysis was performed as previously described.<sup>14</sup> Samples for genome-wide 250k SNP array analysis were processed in accordance with the standard Affymetrix GeneChip protocol (Affymetrix inc, Santa Clara, California, USA) and analyzed using the Copy Number Analyzer for GeneChip (CNAG) v2.0 software package.<sup>48</sup> CNVs were mapped according to the USCS genome browser build March 2006.

### Segregation analysis

Segregation of the detected CNVs was tested in the parents if available. In case of affected family members, segregation of the CNV with the disease was further examined. Segregation was tested by Multiplex Ligation-dependent Probe Amplification (MLPA), Fluorescent In Situ Hybridization (FISH), quantitative Polymerase Chain Reaction (qPCR) experiments or 250k SNP array analysis.

### X- chromosome inactivation (XCI) analysis

XCI analysis was performed whenever an X-CNV inherited from a healthy (or obviously milder affected) mother was identified. XCI patterns were determined using a widely used method based on the presence of a polymorphic repeat within the 5' end of the *Androgen Receptor (AR) gene*, as described by Allen *et al.*<sup>49</sup> A sample was considered to have skewed X-inactivation if the same X chromosome was inactivated in at least 90% of the cells.<sup>50</sup>

### Assessment of clinical relevance

X-CNVs were classified into three different categories reflecting their presumed clinical relevance. The classification categories include:

- 1) (likely) pathogenic CNVs
- 2) CNVs with unknown clinical relevance
- 3) (likely) non-pathogenic CNVs

Table 1 shows a schematic overview of the criteria that were considered to assess clinical relevance and to categorize the X-CNVs into these three classification categories. Classifications of X-CNVs in each individual patient in this cohort were

**Table 1** Criteria for assessment of clinical relevance, grouped by the three different classification categories.

Criteria	Classification		
	<i>(Likely) pathogenic</i>	<i>Unknown</i>	<i>(Likely) non-pathogenic</i>
Inheritance	De novo occurrence	Inherited X-CNV in a male	Paternally inherited X-CNV in female
X-inactivation	Skewed X-inactivation of the affected allele in a healthy carrier mother	Random or unknown X-inactivation pattern in carrier mother	Skewed X-inactivation of the non-affected allele in a healthy carrier mother
Occurrence in patients and controls	Similar CNV in patient(s) with overlapping phenotype No reports in healthy controls	Low detection rate in apparently healthy controls, but higher frequency in individuals with CD and/or CA	Polymorphism in healthy controls No segregation with the phenotype within the family
CNV size and gene content	Size (> 1.5 Mb) and gene content (>20 genes) <sup>17</sup>	Small number of genes that have not been previously related to CD and/or CA	No genes involved
Known association with CD and/or CA of involved genes	Overlap with known CD/CA associated gene	No overlap with known CD/CA associated gene	
Expression and function of involved genes	Role in brain development and/or functioning Brain specific expression of involved gene(s)	Unknown function and/or expression pattern of the gene	Non CD and/or CA related gene function Not expressed in brain
Mouse models	Signs of central nervous system disorder and/or lethal phenotype in knockout mouse	Unknown phenotype in mouse	No phenotype in knock out mouse

CD= cognitive disorders CA=congenital anomalies

based on careful consideration of the total combination of the criteria for assessment of clinical relevance as mentioned in Table 1. None of the criteria was absolute and each criterion was considered in perspective to the other criteria, the currently available information in literature and databases, and the phenotype of the patient.

Results

General overview

In total 57 X-CNVs were analyzed. Tables 2-4 summarize (likely) pathogenic X-CNVs, CNVs with unknown clinical relevance and (likely) non-pathogenic CNVs, respectively. Concise information about the phenotype is included as well. X-CNVs that had initially been identified by conventional karyotyping and were further delineated by SNP array analysis, microscopically visible X-CNVs, and X-CNV gains of the pseudo-autosomal region (PAR) indicating an XXY or XYY karyotype, were excluded from the analysis. The total number of X-CNVs comprised 57 in 4,407 (1.3%), including 22 losses and 35 gains. CNV size ranged from 10 kb to 8.5 Mb.

Twenty-six percent of these (15/57, 7 gains, 8 losses) were classified as pathogenic, and 42% (24/57) were classified as non-pathogenic (16 gains, 8 losses). In 18 cases we could not make a decision about the pathogenicity (12 gains, 6 losses). This gives a detection rate of approximately 0.3% pathogenic X-CNVs in our cohort, that noteworthy consists of patients not preselected on the suspicion of an X-linked disorder. ID was reported in 93% (14/15) of the patients with a pathogenic CNV. The male to female ratio of patients with an X-CNV in this cohort is 1.5:1, compared to a 1.2:1 male to female ratio in the total of our cohort. The male to female ratio of patients with a *pathogenic* X-CNV is with a ratio of 6.5:1 significantly higher (13 males and 2 females). The inheritance of 40 (70%) X-CNVs was tested. Four out of 40 (10%) had occurred *de novo* (all pathogenic X-CNVs). Thirty-four of 36 inherited X-CNVs were maternally inherited (94%). Nine out of 15 pathogenic CNVs (60%) were maternally inherited. In two cases segregation in the grandparents was tested (patients 22 and 51). X-inactivation analysis was performed in 21 out of 34 (62%) carrier mothers. X- inactivation was significantly skewed (at least 90:10) in four of these 21 carrier mothers (19%) (three analyses were uninformative).

**Table 2** X-CNVs that are (likely) pathogenic (N=15).

Number	Sex	Phenotype	CNV	Chromosomal region	Start-End Mb positions (Hg18)	Size/Gene content	Inheritance	XCI	Genes of interest	Disease association	References previously reported overlapping CNVs
1	M	ID, Chondroplasia punctata	Loss	Xp22.33	0.20-3.21	3 Mb /20 genes	Mat	NT	1) <i>SHOX</i> 2) <i>ARSE</i>	1) Leri-Weill dyschondrosteosis (OMIM id 127300); Langer mesomelic dysplasia (OMIM id 249700); short stature, idiopathic, X-linked (OMIM id 300582); 2) Chondroplasia punctata (OMIM id 302950)	Decipher id 978, 2207 <sup>23, 43, 53-55, 72</sup>
2	M	MCA (skeletal)	Loss	Xp22.33	0.14-1.98	2.1 Mb/12 genes	NT	NT	1) <i>SHOX</i>	1) see point 1) above	See above
3	M	Moderate-severe ID, short stature, hydrocephaly	Loss	Xp22.31p22.33	0.14-6.41	6.4 Mb/20 genes	Mat (Mat learning difficulties)	UI in mother Random in affected sister (4572)	1) <i>SHOX</i> 2) <i>ARSE</i> 3) <i>NLG4</i>	1) see point 1) above 2) see point 2) case 1506 3) Autism, X-linked, susceptibility to, 2, mental retardation X-linked included (OMIM id 300495)	See above
4	M	ID, Kallman syndrome, ichthyosis	Loss	Xp22.31p22.33	0.14-8.67	8.5 Mb/37 genes	NT	NT	1) <i>SHOX</i> 2) <i>ARSE</i> 3) <i>NLG4</i> 4) <i>STS</i> 5) <i>KAL1</i>	1-3) see above 4) X-linked ichthyosis (OMIM id 308100) 5) Kallman syndrome (OMIM id 308700)	See above
5	M	DD, ASD, micropenis, ichthyosis	Loss	Xp22.31p22.32	5.20-7.91	2,7 Mb/6 genes	Mat	NT	<i>NLG4</i>	Autism, X-linked, susceptibility to, 2, mental retardation X-linked included (OMIM id 300495)	43, 68-70
6	M	Muscular dystrophy, mild ID	Gain	Xp21.1	32.47-32.71	240 kb /1 gene	Mat	NT	<i>DMD</i>	muscular dystrophy Duchenne type (OMIM id 310200)	60% of patients with Duchenne muscular dystrophy carry a large deletion in <i>DMD</i> <sup>73</sup>
7	F	ID, microcephaly	Loss	Xp11.4p11.3	41.86-45.31	3.5 Mb/9 genes	De novo	Slightly skewed (70:30)*	1) <i>NDP</i> 2) <i>MAOA</i>	1) Norrie disease (OMIM id 310600) 2) Brunner syndrome (OMIM id 300615)	33, 35, 40
8	M	ID, autism, myopathy	Gain	Xq11.2q13.1	62.80-70.52	7.7 Mb/14 genes	Mat (mother mild ID)	Skewed pattern in mother (100:0)*	1) <i>OPHN1</i> 2) <i>EFNB1</i> 3) <i>DLG3</i> 4) <i>MED12</i> 5) <i>ARGHEF9</i>	1) Mental retardation, X-linked, with cerebellar hypoplasia and distinctive facial appearance (OMIM id 300486) 2) Craniofrontonasal dysplasia (OMIM id 304110) 3) Mental retardation, X-linked-90 (OMIM id 300850) 4) Lujan-Fryns syndrome (OMIM id 309520) 5) Epileptic encephalopathy, early infantile, 8 (OMIM id 300607)	

Table 2 Continued.

Number	Sex	Phenotype	CNV	Chromosomal region	Start-End Mb positions (Hg18)	Size/Gene content	Inheritance	XCI	Genes of interest	Disease association	References previously reported overlapping CNVs
9	M	Severe ID, relatively large head circumference, autism	Gain	Xq13.1	69.54-70.11	575 kb/ 6 genes	(G)Mat; cosegregation with disease in male family members	Skewed pattern in mother (80:20)**, random in GM	<i>DLG3</i>	Mental retardation, X-linked 90, MRX90	Tucker <i>et al.</i> reported a larger (2Mb) overlapping gain <sup>31</sup>
10	F	ID, microcephaly, cerebellar vermis hypoplasia	Gain	Xq13.1q13.2	72.15-72.92	500 kb/5 genes	<i>De novo</i>	In patient: not informative	-	Partly overlapping gain in patient with phenotypic overlap, reported by Mc Mullan <i>et al.</i> [22]	Distally overlapping gain in Decipher, id 251339 (ID, microcephaly)
11 (previously reported by Lugtenberg <i>et al.</i> [78])	M	Severe ID, minor facial and genital anomalies, short stature, broad thorax	Gain	Xq13.2q21.1	72.39-79.35	7 Mb/28 genes	<i>De novo</i>	-	1) <i>ATRX</i> 2) <i>MAGT1</i> 3) <i>SLC16A2</i>	1) Alpha thalassaemia mental retardation syndrome (#301040) 2) mental retardation, X-linked 95 (#300716) 3) MCT8 deficiency (#300523)	<sup>40</sup> Decipher id 250180, 251333, 251339, 251751
12	M	Severe ID and neurological problems	Gain	Xq22.1q22.3	99.78-105.33	5.5 Mb/60 genes	Mat (Mat borderline ID)	Skewed pattern in mother (100:0)*	<i>PLP1</i>	Pelizaes Merzbacher disease (#312080)	Decipher id 320, 4480, 249139, 249995, 250543
13 (previously reported by de Leeuw <i>et al.</i> , [74])	M	Moderate-severe ID, microcephaly, VSD, genital, skeletal, intestinal and immunological problems, seizures	Loss	Xq24	118.40-118.60	200 kb/4 genes	Mat	NT	<i>UBE2A</i>	Mental retardation, X-linked, syndromic (OMIM id 312180) <i>UBE2A</i> deficiency syndrome	<sup>74-76</sup>
14	M	Severe ID, short stature, microcephaly	Gain	Xq25q26.2	128.53-133.23	4.68 Mb/28 genes	Mat	Skewed pattern in mother (100:0)*	1) <i>MBNL3</i> 2) <i>GPC3</i> 3) <i>ZDHHC9</i> 4) <i>PDCD8</i>	1) Co-localization of the protein with repeat foci in DM1 and DM2 (Duchenne muscular dystrophy) 2) Simpson-Golabi-Behmel (OMIM id 312870) 3) X-linked syndromic mental retardation (OMIM id 300799) 4) combined oxidative phosphorylation deficiency-6 (OMIM id 300816)	Decipher id 253888 Two overlapping cases with similar phenotypes in personal communication (TK)
15	M	ID, bilateral inguinal hernia, ataxia	Loss	Xq28	147,21-148,54	1.3 Mb/ kb/10 genes	<i>De novo</i>	-	1) <i>FMR2</i> 2) <i>IDS</i>	1) Mental retardation, X-linked, FRAXE type 1 (OMIM id 309548) 2) Hunter syndrome/ Mucopolysaccharidosis II (OMIM id 309900)	Decipher id 250246.

XCI: X-chromosome inactivation UI: uninformative. NT=not tested. NR: not relevant. VSD: ventricular septal defect. P: paternal. Mat: maternal. GP: grandpaternal. GM: grandmaternal. BP: brotherpair.

\* preferential activation of the normal allele \*\* preferential activation of the allele with the CNV.



**Table 3** X-CNVs with unknown clinical relevance (N=18).

Number	Sex	Phenotype	CNV	Chromosomal region	Start-End positions (Hg18)	Size/Gene count	Inheritance	XCI	Genes of interest	Disease association	References previously reported overlapping CNVs
16 (sib of case 3)	F	Moderate-severe ID, hyperactivity, height -2 to -1 SD)	Loss	Xp22.31p22.33	0.14-6.41	6.4 Mb/20 genes	Mat (Mat learning difficulties)	UI in mother Random in patient	1) <i>SHOX</i> 2) <i>ARSE</i> 3) <i>NLGN4</i>	1) Leri-Weill dyschondrosteosis (OMIM id127300); Langer mesomelic dysplasia (OMIM id 249700); short stature, idiopathic, X-linked (OMIM id 300582); 2) Chondroplasia punctata (OMIM id 302950) 3) Autism, X-linked, susceptibility to, 2, mental retardation X-linked included (OMIM id 300495)	Decipher id 978, 2207 23, 43, 53-55, 72, 77
17	M	X-linked ichthyosis, concentration and emotional problems	Loss	Xp22.31	6.51-7.66	1.15 Mb/ 2 genes	NT	NT	<i>STS</i> <i>VCX3A</i>	X-linked ichthyosis (OMIM id 308100). The CNV explains part of the phenotype, but an association with the psychological problems is uncertain	<sup>32, 64-66</sup> Also many cases reported in Decipher. Reported associations with ID are conflicting
18	M	ID, MCA	Gain	Xp22.31	7.04-7.23	190 kb/2 genes	NT	NT	<i>STS</i>	See case 17	See case 17
19	M	X-linked ichthyosis, spastic quadriplegia, ID	Loss	Xp22.31	6.83-7.84	1 Mb/4 genes	NT	NT	<i>STS</i> <i>VCX3A</i>	See case 17 The CNV explains the X-linked ichthyosis, and might contribute to the ID, but an association with the spastic quadriplegia is unlikely	See case 17
20	M	X-linked ichthyosis, skeletal dysplasia, epilepsy, ID	Loss	Xp22.31	6.51-8.12	1.6 Mb/5 genes	Mat	Random pattern in mother	<i>STS</i> <i>VCX3A</i>	See case 17 The CNV explains the X-linked ichthyosis, and might contribute to the ID, but is likely not related to the other symptoms in this patient	See case 17
21	M	Autism, DD, X-linked ichthyosis	Loss	Xp22.31	6.41-8.05	1.6 Mb/6 genes	Mat	NT	<i>STS</i> <i>VCX3A</i>	See case 17. The CNV explains the X-linked ichthyosis, and might explain the DD and autism	See case 17
22	M	ID, hypotonia, nasal lacrimal duct anomaly	Gain	Xp22.31p22.32	5.89-6.41	520 kb/1 gene	Mat+ GP	NT	<i>NLGN4</i>	Autism, X-linked, susceptibility to, 2, mental retardation X-linked included (OMIM id 300495)	<sup>43, 68-70</sup>
23	M	ADHD	Gain	Xp22.2p22.32	9.04-10.02	600 kb/4 genes	Mat	Random pattern in mother	<i>GPR143</i>	Albinism, ocular, type 1 (OMIM id 300500)	-
24	M	ID, extrapyramidal disorder, dysmorphism	Gain	Xp22.2	15.81-16.26	400 kb/1 gene	Mat	Random pattern in mother	<i>GRPR</i>	-	Ishikawa-Brush <i>et al.</i> <sup>56</sup> reported a female patient with 27-year-old female patient manifesting multiple exostoses and autism accompanied by ID and epilepsy and a balanced translocation through <i>GRPR</i>

Table 3 Continued.

Number	Sex	Phenotype	CNV	Chromosomal region	Start-End positions (Hg18)	Size/Gene count	Inheritance	XCI	Genes of interest	Disease association	References previously reported overlapping CNVs
25	M	ID, MCA	Gain	Xp22.2p22.13	17,00-17,47	470 kb/2 genes	Mat	NT	<i>NHS</i>	Nance Horan Syndrome (OMIM id 302350)	Hayashi <i>et al.</i> <sup>33</sup> reported a slightly larger gain in a male patient
26	M	DD, trichonocephaly, UGR, ASD, hypermetropia, asthma	Gain	Xp22.11	22.01-22.11	10 kb/1 gene	Mat	Skewed pattern in mother (80:20)*	<i>PHEX</i>	Hypophosphatemic rickets, X-linked dominant (OMIM id 307800)	In Jaillard <i>et al.</i> <sup>18</sup> 2 brothers with larger overlapping gain are reported. Their phenotype includes ID and microcephaly
27	M	IUD	Loss	Xp21.3	25.75-26.55	800 kb/3 genes	Mat	Skewed pattern in the mother (85:15)*	-	-	-
28	M	DD	Gain	Xp11.4p21.1	37.49-38.26	770 kb/6 genes	Mat	Random pattern in mother	<i>OTC</i>	Involved in ornithine transcarbamylase deficiency (OMIM id 300461). No corresponding metabolic defect in this patient	-
29	M	ID	Gain	Xq22.1	99.41-99.65	250 kb/1 gene	NT	NT	<i>PCDH19</i>	Epilepsy, female-restricted, with mental retardation (OMIM id 300088) Scheffer <i>et al.</i> <sup>78</sup> reported obsessive, rigid personalities in 5 males	-
30	M	Clinical diagnosis of Noonan syndrome	Gain	Xq24	117.5 - 117.7	200kb/1 gene	Mat	Random pattern in mother	-	-	-
31	M	ID, MCA	Gain	Xq28	148.00-148.50	500kb/6 genes	Mat	Slightly skewed pattern (80-20%)**	1) <i>IDS</i> 2) <i>MAGEA9</i>	1) Hunter syndrome/ Mucopolysaccharidosis II (OMIM id +309900) 2) may be implicated in dyskeratosis congenita (Refseq, July 2008)	-
32	M	hypotonia	Gain	Xq28	148.33-148.53	200 kb/5 genes	Mat	Random pattern in mother	1) <i>IDS</i> 2) <i>MAGEA9</i>	See above	-
33	M	ID, MCA	Gain	Xq28	149.16-149.63	480 kb/3 genes	Mat	UI in mother	<i>MTM1</i>	X-linked myotubular myopathy (OMIM id 310400)	-

XCI: X-chromosome inactivation UI: uninformative. NT=not tested NR: not relevant. IUD: intra-uterine death. P: paternal; Mat: maternal; GP: grandpaternal. \* preferential activation of the normal allele \*\* preferential activation of the allele with the CNV.

**Table 4** (Likely) non pathogenic X-CNVs (N=24).

Number	Sex	Phenotype	CNV	Chromosomal region	Start-End positions (Hg18)	Size/Gene count	Inheritance	XCI	Genes of interest	Disease association	References previously reported overlapping CNVs
34	F	ID	Gain	Xp22.32p22.33 In addition: a <i>de novo</i> 8 Mb loss in 1qter	0.14-5.83	5.7 Mb/> 10 genes	NT	NT	1) <i>SHOX</i> 2) <i>ARSE</i> 3) <i>NLGN4</i>	1) Leri-Weill dyschondrosteosis (OMIM id127300); Langer mesomelic dysplasia (OMIM id 249700); short stature, idiopathic, X-linked (OMIM id 300582); 2) Chondroplasia punctata (OMIM id 302950) 3) Autism, X-linked, susceptibility to, 2, mental retardation X-linked included (OMIM id 300495)	Decipher id 978, 2207 <sup>23, 43, 53-55, 72</sup>
35	F	ID, acoustic canal atresia, MCA	Gain	Xp22.33 In addition: 5.1 Mb <i>de novo</i> loss in 18q22.3	0.16-2.41	2.3 Mb/12 genes	NT	NT	<i>SHOX</i>	See above	See above
36	F	ID	Gain	Xp22.33	1.74-3.35	1.5 Mb/8 genes	Mat	In the patient 70:30 X-inactivation pattern** In mother random X-inactivation.	<i>ARSE</i>	See above	See above Overlapping gains are reported in healthy controls
37	M	MCA	Gain	Xp22.33	1.98-3.18	1.1 Mb/9 genes	NT	NT	<i>ARSE</i>	See above	See above
38	F	Severe ID, microcephaly, growth retardation, neuronal migration disorder	Gain	Xp22.31	6.03-6.68	600 kb/ 2 genes	P	NT	<i>NLGN4</i>	See point 3 case 34	43, 68-70
39	F	ID, Costeff syndrome (genetically confirmed)	Gain	Xp22.31	6.5-8.1	1.55 Mb/6 genes	NT	NT	1) <i>STS</i> 2) <i>VCX3A</i>	1) X-linked ichthyosis (OMIM id 308100).	<sup>32, 64-66</sup> Also many cases reported in Decipher. Reported associations with ID are conflicting 2) Deletion of <i>VCX3A</i> was thought to be related to ID because of the gene's absence in XLI patients with ID and its presence in XLI patients without ID. <sup>64</sup>

Table 4 Continued.

Number	Sex	Phenotype	CNV	Chromosomal region	Start-End positions (Hg18)	Size/Gene count	Inheritance	XCI	Genes of interest	Disease association	References previously reported overlapping CNVs
40	F	Severe speech delay	Gain	Xp22.31	6.5-8.1	1.55 Mb/6 genes	NT	NT	STS, VCX3A	See above	See case 39
41	F	DD	Gain	Xp22.31	6.5-8.1	1.55 Mb/6 genes	Mat	NT	STS, VCX3A	See above	See case 39
42	F	ID, progressive cerebellar ataxia, hypotonia	Gain	Xp22.31	7.05-8.1	1.05 Mb/4 genes	Mat	Skewed inactivation (90:10) in the mother*; non informative analysis in patient	STS, VCX3A	See above	See case 39
43	F	ID, epilepsy, alopecia, hearing loss	Loss	Xp22.31	6.5-8.1	1.55 Mb/6 genes	Mat	In patient: UI	STS, VCX3A	See above	See case 39
44	F	Severe ID	Loss	Xp22.31	6.5-8.1	1.55 Mb/6 genes	Mat	NT	STS, VCX3A	See above	See case 39
45	F	Cerebellar ataxia (rombencephalosynapsis), attention and concentration deficits	Loss	Xp22.31	6.5-8.1	1.55 Mb/6 genes	NT	NT	STS, VCX3A	See above	See case 39
46	F	ID, mitochondrial disease	Loss	Xp22.31	6.5-8.1	1.55 Mb/6 genes	NT	NT	STS, VCX3A	See above	See case 39
47	F	ID	Loss	Xp22.31	6.5-8.1	1.55 Mb/ 6 genes	Mat	NT	STS, VCX3A	See above	See case 39
48	F	Abnormal hair, scoliosis	Loss	Xp22.31	6.5-8.1	1.55/6 genes	NT	NT	STS, VCX3A	See above	See case 39
49	F	ID/MCA	Loss	Xp22.31 In addition: 5.3 Mb <i>de novo</i> loss in 7q32.3q33	6.5-8.1	1.55 Mb/6 genes	NT	NT	STS, VCX3A	See above	See case 39
50	F	ID	Loss	Xp22.31	6.5-8.1	1.55 Mb/6 genes	NT	NT	STS, VCX3A	See above	See case 39
51	F	Growth retardation, congenital heart and kidney defects	Gain	Xp21.2	30.30-31.34	1.04 Mb/5 genes	Mat +GP	Uninformative (in patient)	1) DMD 2) GK	1)Duchenne muscular dystrophy 2)Glycerol kinase deficiency (#307030)	-
52	M	ID	Gain	Xp11.4	38.39-38.44	50 kb /1 gene	Mat	NT	TSPAN7	Mental retardation, X-linked 58 (OMIM id 300210)	<sup>58-63</sup>
53	M	ID	Gain	Xp11.4	38.39-38.44	50 kb /1 gene	Mat	Random pattern in the mother	TSPAN7	See above	See above

Table 4 Continued.

Number	Sex	Phenotype	CNV	Chromosomal region	Start-End positions (Hg18)	Size/Gene count	Inheritance	XCI	Genes of interest	Disease association	References previously reported overlapping CNVs
54	F	ID, renal dysgenesis, optical coloboma	Gain	Xq21.1	76.44-77.37	600 kb/ 7 genes	Mat	UI in patient	1) <i>ATRX</i> , 2) <i>ATP7A</i>	1) Alpha thalassaemia mental retardation syndrome(#301040) 2) Menkes disease (#309400)	<sup>40</sup> Decipher id 250180, 251333, 251339, 251751
55	M	Death at 3 months of age, hypertrophic cardiomyopathy	Gain	Xq26.2	130.44-130.80	360 kb/2 genes	Mat	NT	-	-	-
56	M	MCA (hand anomalies)	Gain	Xq26.2	130.44-130.80	360 kb/2 genes	NT	NT	-	-	-
57	F	Severe ID, ASD	Gain	Xq27.1q27.2 In addition: 1 Mb <i>de novo</i> gain in 18q21.2 ( <i>TCF4</i> )	140.05-140.38	330 kb/2 genes	P	NT	-	-	-

XCI: X-chromosome inactivation UI: uninformative. NT=not tested NR: not relevant. P: paternal. Mat: maternal; GP: grandpaternal. \* preferential activation of the normal allele \*\* preferential activation of the allele with the CNV.

In one carrier mother we observed preferential inactivation of the normal allele. In all four carrier mothers with significant skewed X-inactivation, the allele with the X-CNV was preferentially inactivated. In one carrier mother we noticed a slightly skewed pattern with preferential activation of the allele carrying the X-CNV inherited by her son (patient 25). In eight female patients X-inactivation was performed. None of them had a skewed X-inactivation (five analyses were uninformative).

Twenty-six X-CNVs (46%) comprised together 31 genes which have been related to X-linked CD and/or CA in previous reports, or are known X-linked genes associated with CD and/or CA, including *ZNF41*, *ZNF81*, *SYN1*, *TSPAN7*, *OTC*, *GK*, *DMD*, *GRPR*, *NHS*, *NLGN4*, *OPHN1*, *DLG3*, *ATRX*, *SLC16A2* (*MCT8*), *MAGT1*, *ATP7A*, *PCDH19*, *PLP1*, *IDS*, *ARSE*, *SHOX*, *FMR2*, *UBE2A*, *GPC3*, *ZDHHC9*, *PDCD8*, *NDP*, *MAOA*, *EFNB1*, *MED12* and *ARGHEF9*.

(Likely) pathogenic CNVs

*De novo* 3.5 Mb loss in Xp11.4p11.3 (case 7)

This X-CNV was detected in a 2-year old female with DD, microcephaly and facial dysmorphic features. The CNV comprises 2 genes that are implicated in ID syndromes,

including *NDP* (Norrie disease, OMIM ID: 310600) and *MAOA* (Brunner syndrome, OMIM ID: 300615). The phenotype of this patient is not specific for neither of these syndromes, nevertheless, these genes might have contributed to the phenotype as well as the other involved genes. Overlapping deletions are more often reported in patients with ID, including one female case.<sup>33, 35, 40</sup>

575 kb Maternally inherited gain in Xq13.1 (case 9)

This gain comprises *Discs*, *Large Homolog 3* (*DLG3*). This gene has previously been associated with ID.<sup>51-52</sup> Though these cases comprise loss-of-function mutations, we classified this gain as pathogenic, since we demonstrated cosegregation with the ID phenotype in the affected males from this family. The mother of the index male had a slightly skewed inactivation of the X-chromosome that was *not* inherited by her son, which may be explained by the detection of a loss comprising seven exons of the *Dystrophin* (*DMD*) gene on her other X-chromosome. A larger (2 Mb) gain was reported by Tucker *et al.*<sup>31</sup>



**500 kb De novo gain in Xq13.1q13.2 (case 10)**

This gain was detected in a 5-year old female patient with severe ID, microcephaly and structural anomalies of the cerebellum on brain MRI. The region does not comprise genes known to be involved in CD and/or CA, nevertheless, the *de novo* occurrence suggests that an association with the ID phenotype is likely. Furthermore, two overlapping cases have been reported. McMullan *et al.*<sup>22</sup> reported a slightly smaller overlapping gain in a female patient with severe ID, microcephaly, hypotonia and cerebral atrophy. In Decipher a distally overlapping gain in a male patient with ID and microcephaly is reported (DECIPHER ID: 251339).

**4.7 Mb maternally inherited gain in Xq25q26.2 (case 14)**

We classified this gain identified in a male patient with ID as pathogenic. Arguments were the relatively large CNV size, involvement of several genes that are implicated in ID phenotypes and a 100% skewed X-inactivation pattern in the mother, with preferential inactivation of the allele with the duplication. The mother had a normal intellect, but a relatively short stature and head circumference.

Furthermore, we know of two families with overlapping X-CNVs in this region. The male cases have similar phenotypes including severe ID, growth retardation and microcephaly. Female carriers had a variable and milder phenotype including normal to mild ID and short stature (personal communication TK).

**1.3 Mb de novo loss in Xq28 (case 15)**

In the Decipher database several, mostly larger overlapping cases are reported. Decipher case number 250246 comprises a 3.5 Mb *de novo* deletion in a male patient with ID, microcephaly and hypotonia. Both the present deletion and the deletion reported in the Decipher database involve the *Fragile X Mental Retardation 2* gene (*FMR2*), which is a likely explanation for the observed ID phenotype.

**CNVs with unknown clinical relevance****6.4 Mb familial deletion in Xp22.31p22.33**

In a brother (case 3) and a sister (case 16) both with moderate-severe ID we identified a maternally inherited Xpter deletion. The mother had mild learning disabilities. FISH studies on blood lymphocytes excluded a mosaic in this tissue. The phenotype of the brother is likely explained by this deletion, but his sister has a relatively severe level of ID for a female and compared to her mother who has an identical deletion. Previously reported females had normal mental performance or mild learning difficulties, a Turner syndrome neurocognitive profile (selective non-verbal deficits) and short to low normal stature. Female carriers might be protected from a severe phenotype by a favorable skewed X-inactivation pattern.<sup>53, 54, 55</sup> It might be speculated that the severe phenotype in the sister is explained by her random

X-inactivation pattern and that the mild phenotype in the mother might be due to a skewed X-inactivation pattern. X-inactivation analysis in the mother was uninformative, so we were not able to confirm this. We cannot exclude that in the severely affected sister other factors contribute to her phenotype as well.

**400 kb Maternally inherited gain Xp22.2 (case 24)**

This gain comprises the gene *gastrin-releasing peptide receptor (GRPR)*. *GRPR* is only once reported in relation to ID and ASD. A translocation disrupting this gene was identified in a single female with ID and autism<sup>66</sup>, but additional evidence for involvement of *GRPR* in ID and/or ASD is lacking. Though, we cannot exclude an association of the duplication of this gene with the observed phenotype in this male patient.

**470 kb Maternally inherited gain in Xp22.13p22.2 (case 25)**

In patient 25 a maternally inherited duplication comprising the *Nance Horan Syndrome gene (NHS)* was detected. This syndrome is associated with a syndromic ID phenotype. However, a causal relation with the ID observed in this patient is uncertain since he does not show the other typical manifestations of Nance-Horan-Syndrome (OMIM ID: 302350). Moreover, this CNV includes a duplication of the region where *NHS* is located, while Nance-Horan syndrome causing mutations are loss-of-function mutations.

**10 kb Maternally inherited gain in Xp22.11 (case 26)**

The breakpoints of this gain disrupt the *phosphate-regulating endopeptidase gene (PHEX)*, which is involved in X-linked dominant hypophosphatemic rickets (OMIM ID: 307800). This disease is not associated with ID and patient 26 does not show signs of the disease. We still classified this CNV as unknown, because Jaillard *et al.*<sup>18</sup> described two brothers who had an overlapping gain and overlap in phenotype. The authors concluded that dosage effects of *PHEX* may be involved in their phenotype. This was based on the observed phenotype in mice with *PHEX* duplications, including behavior and craniofacial abnormalities.<sup>57</sup> The gain in the brothers reported by Jaillard *et al.*<sup>18</sup> comprised in addition the gene *ZNF645*. An effect of duplication of this gene in the phenotype of those brothers cannot be excluded.

**250 kb Gain in Xq22.1 (case 29)**

This gain comprises the gene *Protocadherin 19 (PCDH19)*. Loss-of function mutations in *PCDH19* are involved in a female restricted phenotype, Epilepsy and mental retardation limited to females (EFMR) (OMIM ID: 300088). This gain was detected in a male patient with ID, but without epilepsy. It cannot be excluded that

an opposite dosage effect of *PDCH19* is the explanation for the phenotype in this male patient. Unfortunately segregation analysis in the mother was not possible.

### **(Likely) non pathogenic X-CNVs**

#### **50 kb Maternally inherited gain in Xp11.4 (cases 52-53)**

This gain comprises the gene *Tetraspanin 7* (*TSPAN7*). In several reports aberrations of *TSPAN7* have been associated with ID.<sup>58-62</sup> Though, Froyen *et al.*<sup>35</sup> reported an ID family with a duplication involving *TSPAN7*, in which the duplication did not cosegregate with the ID phenotype. Furthermore, Cai and colleagues<sup>63</sup> identified two *TSPAN7* duplications in 174 healthy male controls. Therefore, we classified the gains of Xp11.4 comprising *TSPAN7* as non-pathogenic.

#### **600 kb Maternally inherited gain in Xq21.1 (case 54)**

This gain was detected in a female patient and comprises two disease associated genes, including *ATRX* (Alpha thalassaemia mental retardation syndrome, OMIM ID: 301040) and *ATP7A* (Menkes disease, OMIM ID: 309400). Because involved genes are involved in X-linked recessive diseases with a different phenotype, and the gain was inherited from a healthy mother we assigned this gain to the (likely) non pathogenic group.

#### **Patients with additional autosomal CNVs that explain the phenotype**

Patients 34, 35, 49 and 57 had an additional autosomal CNV being the most likely explanation for their phenotypes.

### **Recurrent gains and losses**

#### **Chromosomal region Xp22.31 (cases 17-21 and 39-50)**

This Xp region has a high frequency of interstitial CNVs due to non allelic homologous recombination (NAHR). In this cohort we detected as many as 17 CNVs in this region (12 losses and 6 gains), suggesting that CNVs in this region are extremely frequent. Five of the CNVs in this region occurred in male patients.

The region comprises several genes, including *Steroid Sulfatase isozyme S* (*STS*) and *VCX3A*. Haploinsufficiency of *STS* causes X-linked recessive ichthyosis (XLI) (OMIM id 308100). Deletion of *VCX3A* was thought to be related to ID because of the gene's absence in XLI patients with ID and its presence in XLI patients without ID.<sup>64</sup> However, a subsequent study detected deletions encompassing *VCX3A* in 62 XLI patients without ID, which is in conflict with a causative relation of deletion of *VCX3A* with ID.<sup>65</sup> Furthermore, the Database of Genomic Variants includes many genomic variants, comprising small copy number variants (mainly gains) in which *VCX3A* is involved. A recent study reported a frequency of 0.37% of the Xp22.31 duplication in a cohort of patients, both males and females, with DD, ID, autism, dysmorphic

features and/or MCA, compared to a frequency of 0.15% in a healthy control population. These data suggest a potential causal relationship of the duplication with the abnormal phenotype, possibly an additive effect.<sup>66</sup> Nevertheless, we classified several CNVs in this region in female patients as non-pathogenic, because of a severe and/or syndromic phenotype that is likely not explained by this X-CNV. In the male cases we classified these CNVs as CNVs with unknown clinical relevance.

#### **Chromosomal region Xp22.31p22.32 (cases 5, 22 and 38)**

Six X-CNVs comprised the region where *NLGN4* is localized. Three of these were larger deletions comprising several genes and corresponding phenotypes are likely the result of contiguous deletion of these genes. Three of these X-CNVs (cases 5, 22 and 38) were smaller and included *NLGN4* as the only candidate gene for the phenotype. In patient 5, a 10-year-old male with ASD as the main presenting feature, a 2.7 Mb deletion involving *NLGN4* was found. The phenotype included mild DD, and ichthyosis as well. Loss-of-function mutations in this gene were previously associated with ASD.<sup>67</sup> Deletions comprising *NLGN4* are more often described and are associated with a variable phenotype comprising both ID, and ASD. Some patients have a normal cognitive performance.<sup>43, 68-70</sup>

In two patients (cases 22 and 38) copy number *gains* involving *NLGN4* were detected. Most previous reports concern deletions and loss-of-function mutations. Though, Daoud *et al.*<sup>71</sup> reported the detection of *de novo* mutations in the promoter region of the gene leading to increased expression in patients with ID and autism. The gains in our patients segregated in unaffected males, but we still classified the gain of patient 22 as unknown, because a variable phenotypic spectrum including normal intellectual function has been reported for the deletions<sup>54</sup>, so we cannot exclude that gains may lead to a similar broad spectrum as well. The gain of patient 38 was classified as (likely) non-pathogenic, because this concerns a very severe phenotype in a female with a healthy father who carries an identical gain.

## **Discussion**

In a large cohort of patients with CD and/or CA examined by genome-wide array analysis in a diagnostic setting, we demonstrated a 1.3% detection rate of X-CNVs. Genome-wide array platforms are now widely being used in the clinical diagnostic setting. As a result, X-CNVs are frequently being detected in routine diagnostics. A careful interpretation of these X-CNVs is essential for adequate counseling of patients and their families. The interpretation of the clinical relevance of X-CNVs is challenging and complicated by many factors. We classified the X-CNVs in our

cohort in three categories of clinical relevance, thereby making use of our own well-defined criteria, previous literature and database reports and the current insights and knowledge. Our report could serve as a resource for clinicians and laboratory specialist in the interpretation of X-CNVs that are detected by genome wide array platforms in a routine diagnostic setting. However, it is obvious that in individual patients the assessment of the clinical relevance of a certain X-CNV should always be made on solid grounds, taking into account the individual situation of the patient -with respect to specific information on phenotype and family history for example- and up-to-date insights and knowledge.

Based on our classification the detection rate of pathogenic X-CNVs in our cohort was 0.3%. The detection rates of X-CNVs with potential/ unknown clinical relevance and non-pathogenic clinical relevance were 0.4% (18/4,407) and 0.5% (24/4,407) respectively. Our results are likely influenced by the relatively poor probe coverage of the X-chromosome on the 250 k SNP array platform, through which we might have missed small X-CNVs in relatively poor covered regions. For example, *MECP2* duplications, which are frequently found in males with ID and progressive neurological symptoms, are not being detected by the array platforms we used, which is of course a serious limitation of these specific platforms.

Interpretation of clinical relevance was mainly based on involvement of known CD and/or CA associated genes in combination with the clinical phenotype. Some gains comprising known ID genes, we did not classify as pathogenic, because previous reports regarding these genes in relation to ID concerned loss-of-function mutations and/or deletions, rather than a gain-of-function. However, it was shown that duplications and loss-of function mutations involving the same gene can each lead to different ID phenotypes, for example loss-of function mutations in *MECP2* give rise to Rett syndrome and duplications involving this gene give rise to a different ID phenotype without the classical features of Rett syndrome.<sup>42, 47</sup> Therefore, we cannot excluded that the gene dosage independently of whether this is an increase or decrease, may be critical.

In selected cases we performed XCI analysis. These analyses mostly confirmed the initially proposed clinical relevance rather than having a significant contributive impact on the final classification in one of the categories. Moreover, results of XCI analysis were not always informative, due to homozygosity for the (CAG)<sub>n</sub> repeat within the 5' end of the *Androgen Receptor (AR) gene*.

The identification of additional identical or overlapping X-CNVs in patients and/or controls, may shed light on X-CNVs that we classified as unknown or ambiguous variants and may in addition reveal additional X-CNVs associated with CA and/or CA. Moreover, the combination of these data together with data generated by exome and genome sequencing of the X-chromosome, which is now increasingly

being implemented in both research and diagnostics, may contribute to further deciphering of the X-linked genetic base of these disorders.

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### Web Resources

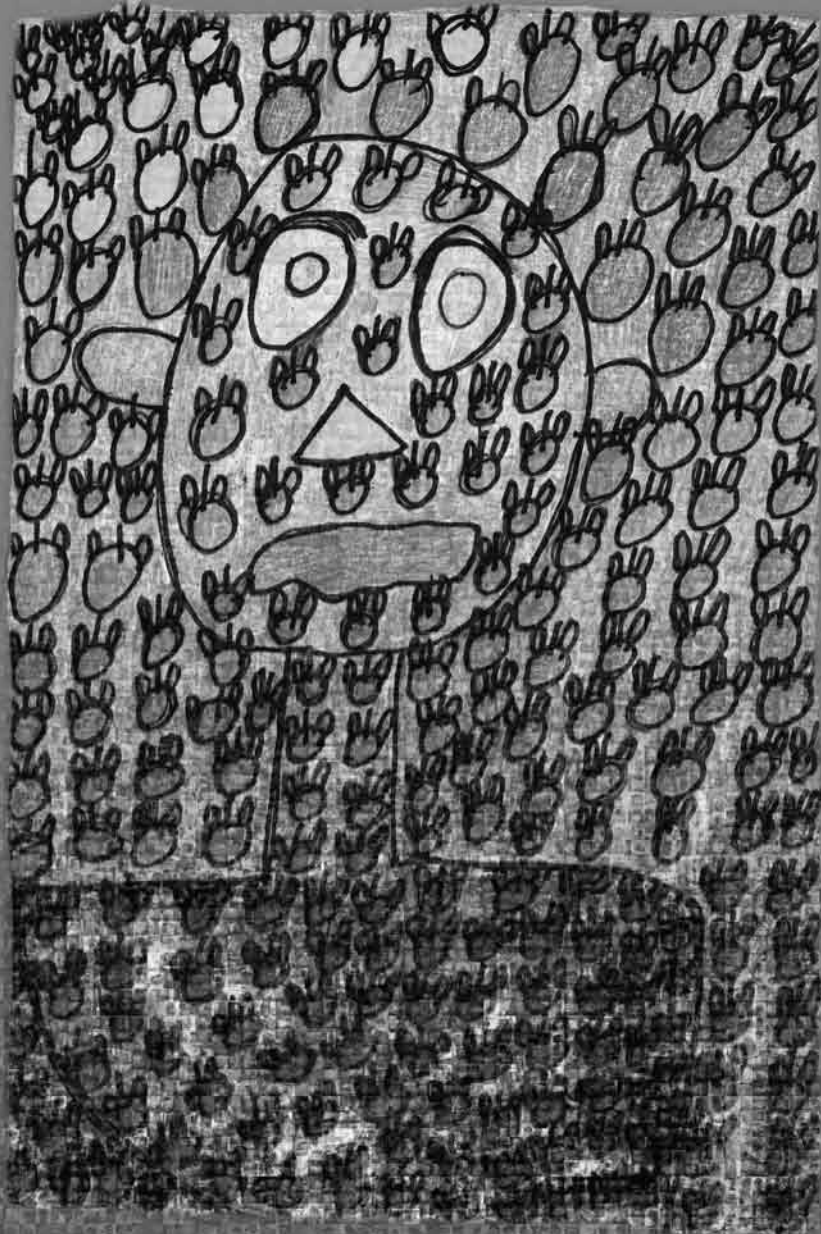
Databases of Genomic Variants: <http://projects.tcag.ca/variation>; Decipher: <http://decipher.sanger.ac.uk>

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# 4

## Further phenotypic delineation of well known ID syndromes

### 4.1 Kleefstra syndrome

4.1.1 Update on Kleefstra syndrome

*Mol Syndromol* 2012;2:202-212

4.1.2 Familial Kleefstra syndrome due to maternal somatic mosaicism for interstitial 9q34.3 microdeletions

*Clin Genet* 2011;80:31-38

### 4.2 Adult phenotypes in Angelman- and Rett-like syndromes

*Mol Syndromol* 2012;2:217-234





# 4.1.1

## Update on Kleefstra syndrome

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## Abstract

Kleefstra syndrome is characterized by the core phenotype of developmental delay/ intellectual disability, (childhood) hypotonia and distinct facial features. The syndrome can be either caused by a microdeletion in chromosomal region 9q34.3 or by a mutation in the euchromatin histone methyltransferase 1 (*EHMT1*) gene. Since the early 1990s, 85 patients have been described, of which the majority had a 9q34.3 microdeletion (>85%). So far, no clear genotype-phenotype correlation could be observed by studying the clinical and molecular features of both 9q34.3 microdeletion patients and patients with an intragenic *EHMT1* mutation. Thus, to further expand the genotypic and phenotypic knowledge about the syndrome, we here report 29 newly diagnosed patients, including 16 patients with a 9q34.3 microdeletion and 13 patients with an *EHMT1* mutation, and review previous literature. The present findings are comparable to previous reports. In addition to our previous findings and recommendations, we suggest cardiac screening during follow-up, because of the possible occurrence of cardiac arrhythmias. In addition, clinicians and caretakers should be aware of the regressive behavioral phenotype that might develop at adolescent/adult age and seems to have no clear neurological substrate, but is rather a so far unexplained neuropsychiatric feature.

## Introduction

Kleefstra syndrome (KS) (OMIM 610253), previously named 9q subtelomeric deletion syndrome (9qSTDs), is characterized by the core clinical phenotype of mostly moderate to severe developmental delay (DD)/intellectual disability (ID), (childhood) hypotonia and distinct facial features, comprising brachy(micro)cephaly, synophrys, unusual shape of the eyebrows, midface hypoplasia, full everted lower lip, cupid bowed upper lip, protruding tongue and prognathism. Additional clinical features include congenital heart and urogenital defects, epilepsy, behavioral and psychiatric disorders and overweight. The syndrome is either caused by a submicroscopic deletion in the chromosomal region 9q34.3 or an intragenic mutation of the euchromatin histone methyltransferase 1 (*EHMT1*) gene causing haploinsufficiency of *EHMT1*. So far 85 patients, including 75 patients with a 9q34.3 deletion and 10 patients with an *EHMT1* mutation have been reported.<sup>1-16</sup> *EHMT1* encodes a histone H3 Lys 9 methyltransferase and is thereby involved in chromatin remodeling.<sup>17</sup> Among the genetically confirmed cases the majority of the patients have a deletion and the remaining a mutation in *EHMT1*.<sup>11, 18</sup> Almost all cases are sporadic, but recently we described 3 familial cases due to a subtelomeric 9q deletion present in mosaic pattern in the mothers.<sup>16</sup> Since the identification of *EHMT1* as the major gene<sup>13</sup>, an increasing number of mutations in this gene is being detected in patients with the core phenotype of KS. Genotype-phenotype studies in patients collected so far indicated that patients with an *EHMT1* mutation and those with a 9q34.3 deletion smaller than 3 Mb have highly comparable clinical findings.<sup>10, 19</sup> Here we report 29 additional diagnosed patients with KS that have not been published before. Sixteen cases were caused by a submicroscopic 9q34.3 deletion and 13 cases by an intragenic *EHMT1* mutation. We describe the clinical and molecular characteristics of this additional cohort, provide an overview of previous patients and compare present and previous findings.

## Patients and Methods

A subset of the patients included in this study was diagnosed at our diagnostic center after referral from our outpatient clinic or from clinical geneticists from national/international collaborating centers. Others were referred to our diagnostic center for follow-up (studies) after the diagnosis had been established elsewhere. The cohort included 16 patients with a 9q34.3 deletion and 13 patients with an intragenic *EHMT1* mutation.

DNA was obtained from peripheral blood cells and extracted according to standardized procedures. Deletions were detected by routine subtelomeric multiplex ligation-

dependent probe amplification (MLPA) (P070, MRC Holland)/FISH (probes from Vysis) or whole genome array analysis with different platforms (Agilent Technologies and Affymetrix Inc., Santa Clara, Calif., USA). In patients without a deletion in 9q34.3, subsequent gene-specific extensive MLPA analysis and direct sequencing of the coding region of *EHMT1* was performed as described previously.<sup>13</sup> In addition, 2 novel coding exons were sequenced, since the recent identification of an elongated *EHMT1* transcript (NM\_024757.4).<sup>20</sup>

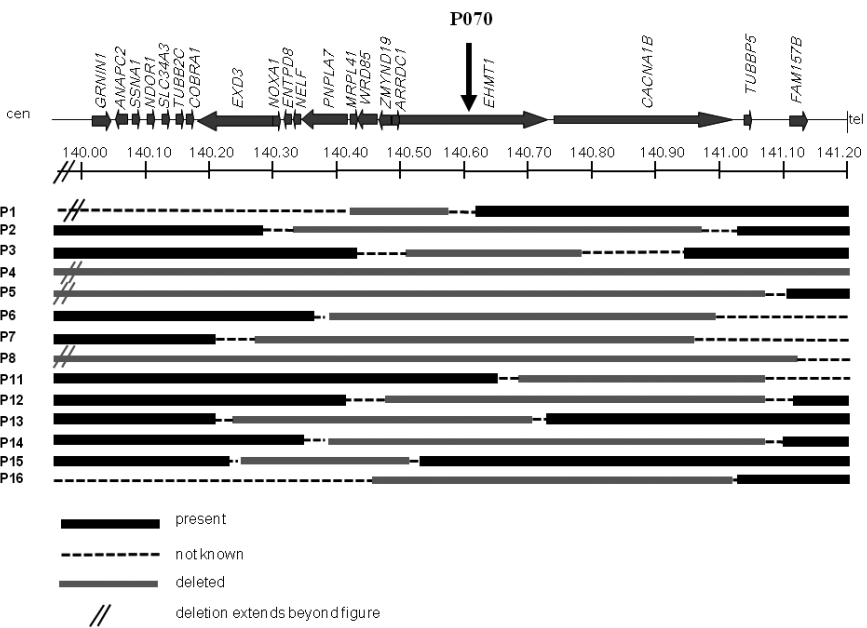
Results

Molecular Data

Figure 1 shows a schematic overview of the deleted regions in patients 1--8 and 11--16 diagnosed with a 9q34.3 deletion. Five deletions were initially identified by routine subtelomeric MLPA analysis (patients 1--3, 9 and 10). The deletions of patients 1--3 were fine mapped by additional region-specific MLPA experiments with probes in *EHMT1* and flanking genes, with the most proximal probe in the gene *RAPGEF1*, which is located approximately 6 Mb distant from *EHMT1* and the most distal probe in *CACNA1B*. The deletion of patient 1 was shown to have a minimum size of 200 kb and a maximum size of 6 Mb and thus poorly mapped (no probes could be tested in between *RAPGEF1* exon 2 (present) and *MRPL41* exon 1 (deleted)). Deletions of patients 9 and 10 are not shown, because these could not be further fine-mapped either. The deletions of patients 4--8 and 11--16 were identified and delineated by different genome wide array platforms, including the Affymetrix 250K SNP array (patients 5 and 16), 2.7M array (patient 4) and 6.0 SNP array platforms (patients 13, 14 and --15), and the Agilent 60K (patient 7), 105K (patients 11 and 12) and 180K oligoarray (patients 6 and 8) platforms. Mb-positions were converted to UCSC genome browser build February 2009 (Hg 19). Present deletions vary in size from 270 kb (patient 15) to 3.85 Mb (patient 4).

*EHMT1* mutations of the present and previously reported patients are shown in figure 2. Present mutations found in the present group are summarized in table 1 as well. The mutations in the present group and include 6 frameshift mutations, 3 nonsense mutations (2 identical: p.Arg620X), 2 exon deletions, 1 missense mutation and 1 mutation in a donor splice site. In 5 patients *de novo* occurrence of the mutation was confirmed by segregation analysis in the parents (patients 21, 22, 24, 25 and 27). In the remaining cases, DNA of one or both parents was not available. Two mutations occurred in the Su(var)3--9, Enhancer-of-zetste, Trithorax (SET) domain (patients 22 and 26), 4 in the preSET domain (patients 19, 25, 27 and patient 28 for whom clinical data was not available), 2 in the ankyrin repeats (ANK) domain 7 (patients 18 and 21), 1 affected the donor splice site in exon 22 (patient 20),

Figure 1 Schematic overview of deleted regions in 9q34.3 in patients 1--8 and 11--16.



The relative positions of involved genes and the P070 MPLA probe are indicated (UCSC genome browser build February 2009, Hg 19). Deletions of patients 1--3 were identified by MLPA. Deletions of patients 4--8 and 11--16 were detected by genome-wide array analysis. The deletion of patient 4 was identified by 2.7M array, the deletion of patients 5 and 16 by 250K SNP array, the deletions of patients 6 and 8 by 180K oligoarray, the deletion of patient 7 by 60K oligoarray and the deletions of patients 11 and 12 by 105K oligoarray. In patients 13--15 the Affymetrix 6.0 SNP array platform was used. Deletions of patients 9 and 10 are not depicted, because these were poorly delineated.

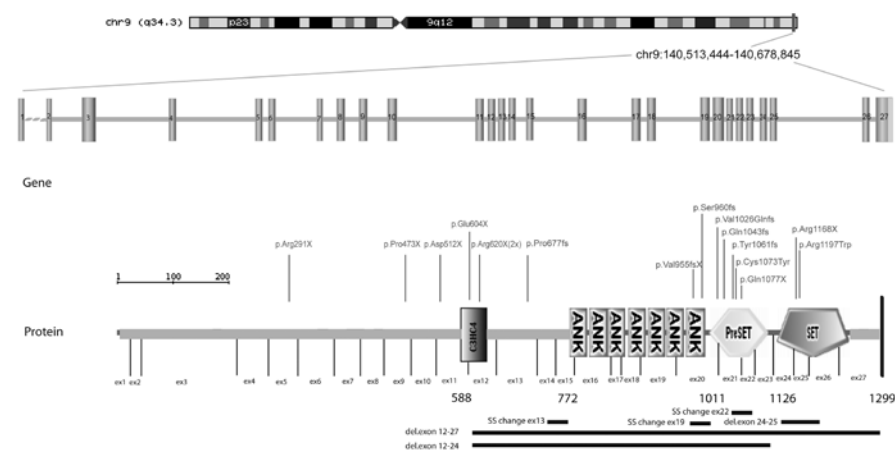
2 mutations (the recurrent p.Arg620X) occurred in the C3HC4 type zinc-finger (RING finger) (C3HC4) domain (patient 17 and patient 29 without available clinical data) and 1 mutation was localized outside the characterized domains (patient 23). The intragenic deletion of patient 24 comprised all characterized domains.

Clinical Data

The clinical features of the patients in this study are summarized in table 2 (9q34.3 deletions) and table 3 (*EHMT1* mutations). Information on the clinical features of the patient with the p.Gln1043fs mutation (patient 28) and one of the patients with the



**Figure 2** Schematic diagram of the *EHMT1* gene structure (including 27 exons) and the protein domain structure of the EHMT1 protein.



The conserved domains include the C3HC4 type zinc-finger (RING finger) (C3HC4) domain, 7 ankyrin repeat (ANK) domains, the Su(var)3--9, Enhancer-of-zetste, trithorax (SET) domain and the PreSET domain (N-terminal to the SET domain).The relative positions of in total 22 mutations are displayed, including the 13 mutations of the present patient cohort (1 recurrent mutation (p.Arg620X), present in 2 patients) and 9 previously reported mutations. The first identified intragenic *EHMT1* aberration due to a translocation X;9 is not shown. Del, deletion; SS, splice site mutation

p.Arg620X mutation (patient 29) was not available and therefore not included in tables 3 and 4. Clinical features were systematically obtained by a standardized clinical data sheet. The age of patients with a microdeletion ranged from 1 to 25 years (average age 9.5 years/median age 5.5 years, including 3 adults and 13 children) and from 1 to 41 years among patients with an *EHMT1* mutation (average age 11.8 years/median age 7.5 years, including 2 adults and 9 children). All patients had the core KS phenotype, including DD/ID, childhood hypotonia and distinct facial features. Clinical photographs of patients with a submicroscopic 9q34.3 deletion are shown in figure 3. Patients with *EHMT1* mutations are shown in figure 4. Patient 20 with an *EHMT1* mutation is described in more detail by Willemsen *et al.*<sup>21</sup> Among the patients with a deletion, the degree of ID ranged from mild to severe, but the majority of the patients had severe ID. Five patients were not yet able to walk (patients 1, 3, 4, 7 and 9 at 5 years, 33 months, 5 years, 12 months and 16 months of age, respectively). Most patients, except for patients 4, 5, 8, 10 and 16, developed some speech, though mostly very primitive (single words). One patient had a

**Table 1** Intragenic *EHMT1* mutations identified in patients 17–29

Patient	Mutation NM_024757.4	Alteration NP_079033.4
17	c.1858C>T	p.Arg620X
18	c.2863_2864del	p.Val955fsX221
19	c.3229C>T	p.Gln1077X
20	c.3180+1G>T	(donor splice site)
21	c.2878_2881	p.Ser960GlyfsX7
22	c.3375-?_3541+?del	(exon 24+25 deletion)
23	c.2029insG	p.Pro677fs
24	exon 12–27 deletion	
25	c.3181-80_3233del	p.Tyr1061fs
26	c.3589C>T	p.Arg1197Trp
27	c.3072_3073del	p.Val1026fs
28	c.3126_3127del	p.Gln1043fs
29	c.1858C>T	p.Arg620X

remarkably mild phenotype and was even able to read and write (patient 13). In the cohort of patients with an intragenic *EHMT1* mutation cognitive performance varied from mild ID with speaking in full sentences and ability to read and write (patient 24) to moderate ID with regression at adult age (patients 20 and 25). Speech ability ranged from a few single words to long sentences with a vocabulary of more than 100 words (patients 23 and 24). All were able to walk. Microcephaly was present in 8 out of 14 patients with a microdeletion and in 2 out of 11 patients with an intragenic *EHMT1* mutation. Overweight (body mass index (BMI) >25) was reported among 3 of 15 patients with a deletion and was more frequent among patients with a mutation (4 of 10). Reported heart anomalies were mainly structural defects, including atrial septal defects, ventricular septal defects, valve anomalies comprising pulmonic valve stenosis and bicuspid aortic valve, persistent open foramen ovale and ductus arteriosus anomalies. One patient (patient 20) was diagnosed with atrial flutter and 1 patient had an aberrant muscle bundle in the left ventricle (patient 14). Genital defects were only observed in males and included cryptorchidism, hypospadias and micropenis (in 5 of 8 males with a microdeletion and in 1 out of 3 males with a mutation, respectively). Musculoskeletal anomalies were reported in 4 of 16 patients with a microdeletion and in 3 of 11 patients with a mutation, respectively, and comprised joint hypermobility, scoliosis



**Table 2** Clinical features of present and previous patients with a 9q34.3 deletion.

Clinical features	Present patients (corresponding photographs in figure 3)																Total	Previous
	1 (A, B)	2 (C–E)	3 (no)	4 (F, G)	5 (H, I)	6 (J, K)	7 (L)	8 (M–O)	9 (no)	10 (P, Q)	11 (R–U)	12 (V)	13 (no)	14 (no)	15 (no)	16 (no)	n = 16 (%)	n = 75 (%)
Deletion size (Mb)	0.2–6	~0.6	0.27–0.41	3.85	1.2	0.6	0.7	3.1	STD	STD	0.4	0.6	0.5	0.7	0.27	0.56–1.32	0.2–6	0.04–CV
Age (years, mo)	5	6	2 9/12	5	11	2	1	10	1 4/12	25	5	5	14	15	25	18	1–25	0–59
Gender	M	M	F	F	F	F	F	M	M	F	M	F	M	M	F	M	8 M, 8 F	M = F
Growth parameters																		
High birth weight	–	+	–	–	NR	–	–	–	–	–	–	–	–	+	–	–	2/15 (13)	4/51 (8)
Microcephaly	+	–	+	NR	+	NR	+	–	+	+	+	+	–	–	–	–	8/14 (57)	24/50 (48)
Short stature	+	–	–	NR	–	–	–	–	–	+	–	–	–	–	–	–	2/15 (13)	16/41 (39)
Overweight (BMI >25)	–	–	–	NR	–	–	–	+	–	–	–	–	–	+	+	–	3/15 (20)	15/49 (31)
DD/ID	sev.	mild	sev.	sev.	sev.	mild	yes	sev.	mild	sev.	mod.	yes	mild	sev.	mod.	sev.	16/16 (100)	75/75 (100)
Heart defect	–	–	–	+	+	–	–	–	+	–	–	–	–	+	+	–	5/16 (31)	22/50 (44)
Genital anomaly	–	+	–	–	–	–	–	–	+	–	+	–	+	–	–	+	5/8 (63)	12/45 (27)
Renal anomaly (including VUR)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0/16 (0)	7/43 (16)
Recurrent infections	–	–	–	+	–	–	+	+	–	–	–	–	–	–	–	+	4/16 (25)	5/19 (26)
Hearing deficit	–	+/-	–	–	–	–	–	–	+	+	+	–	+	–	–	–	5/16 (31)	7/36 (19)
Gastro-esophageal reflux	–	+	–	–	+	–	+	+	–	–	–	–	–	+	–	+	6/16 (38)	4/36 (11)
Epilepsy	–	–	–	+/-	–	–	–	–	–	–	+	+	+	+	+	+	7/16 (44)	14/43 (33)
Behavioral/psychiatric	–	+	+	+	+	–	–	+	–	+	+	+	–	–	+	+	10/16 (63)	22/43 (51)
Anomalies on brain imaging	NR	NR	–	+	+	NR	NR	–	NR	NR	–	+	+	+	–	–	5/10 (50)	20/33 (61)
Tracheomalacia	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0/16 (0)	6/37 (16)
Umbilical/inguinal hernia	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	+	3/16 (19)	6/38 (16)
Anal atresia	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0/16 (0)	2/51 (4)
Musculoskeletal anomaly	–	–	–	+	+	–	–	–	–	–	+	–	–	–	–	+	4/16 (25)	9/35 (26)
Respiratory complications*	–	–	–	+	–	–	+	–	–	+	–	–	–	–	–	–	3/16 (19)	5/42 (12)
Other	–	–	–	–	T	–	–	E	–	–	–	–	–	–	–	E		

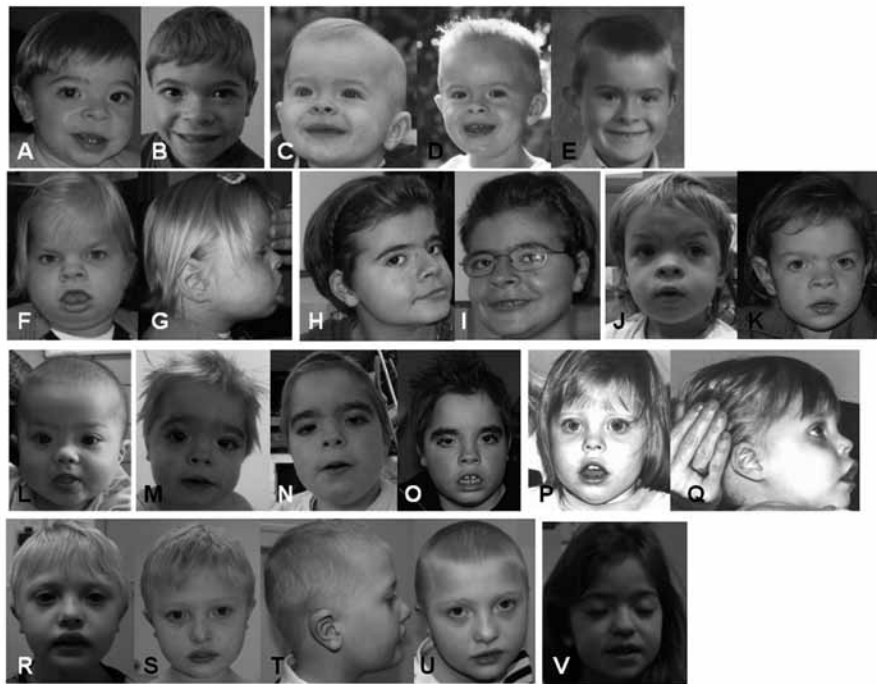
CV, cytogenetically visible; DD/ID, developmental delay/intellectual disability; mild, mild ID; mod., moderate ID; sev., severe ID; yes, ID present (level not known); E, eye anomalies: coloboma in patient 8, severe hypermetropia in patient 16, congenital cataract in patient 23; F, female; M, male; NR, not reported; STD, subtelomeric deletion; T, teeth anomalies; VUR, vesicoureteral reflux. \*Including cardiorespiratory failure, apneas; +, yes; –, no; +/- uncertain

**Table 3** Clinical features of present and previous patients with an intragenic *EHMT1* mutation.

Clinical features	Present patients (corresponding photographs in figure 4)											Total	Previous
	17 (A, B)	18 (C–E)	19 (F, G)	20 (H, I)	21 (J, K)	22 (no)	23 (no)	24 (L–N)	25 (O–R)	26 (S–U)	27 (V, W)	n = 11 (%)	n = 10 (%)
Mutation (NM_024757.4)	p.Arg 620X	p.Val 955fs	p.Gln 1077X	SS exon 22	p.Ser929 GlyfsX	del. exon 24–25	p.Pro 677fs	del. exon 12–27	p.Tyr 1061fs	p.Arg 1197Trp	p.Val 1026fs		
Age (years, mo)	2 2/12	6 2/12	2 9/12	41	5 5/12	7 5/12	2 5/12	9 2/12	32	10	11 5/12	2 9/12–41	2,6–16
Gender	M	F	M	F	M	F	F	F	F	F	F	3 M, 8 F	4 M, 6 F
Growth parameters													
High birth weight	–	–	–	–	–	NR	–	–	+	–	–	1/10 (10)	3/9 (33)
Microcephaly	+	–	–	–	–	–	+	–	–	–	–	2/11 (18)	2/10 (20)
Short stature	–	–	–	–	–	NR	–	–	+	–	–	1/10 (10)	2/8 (25)
Overweight (BMI >25)	–	–	+	+	–	+	–	–	NR	+	–	4/10 (40)	4/9 (44)
DD/ID	yes	mod.	yes	mod.	yes	mild	mild	mild	mod.	yes	mod.	11/11 (100)	10/10 (100)
Heart defect	+	–	+	+	–	–	+	+	–	–	–	5/11 (45)	4/10 (40)
Genital anomaly	+	–	–	–	–	–	–	–	–	–	–	1/3 M (33)	2/4 M (50)
Renal anomaly (including VUR)	–	–	+	–	–	–	–	+	–	–	–	2/11 (18)	1/10 (10)
Recurrent infections	–	+	+	+	+	–	+	–	+	–	+	7/11 (64)	NR
Hearing deficit	–	–	–	–	+	–	–	–	+	+	+	4/11 (36)	1/10 (10)
Gastro-esophageal reflux	–	–	+	–	+	–	–	–	–	–	+	3/11 (27)	0/10 (0)
Epilepsy	+	–	–	–	+	–	–	–	–	–	–	2/11 (18)	3/10 (30)
Behavioral/psychiatric problems	–	+	–	+	+	–	+	+	+	+	+	8/11 (73)	7/9 (78)
Anomalies on brain imaging	–	–	+	+	+	+	NR	+	+	+	–	7/10 (70)	5/9 (56)
Tracheomalacia	–	–	–	–	–	–	–	–	–	–	–	0/11 (0)	1/10 (10)
Umbilical/inguinal hernia	–	–	–	–	–	–	+	–	–	–	–	1/11 (9)	1/10 (10)
Anal atresia	–	–	–	–	–	–	–	+	–	–	–	1/11 (9)	0/10 (0)
Musculoskeletal anomaly	–	–	–	–	–	–	+	+	+	–	–	3/11 (27)	1/10 (10)
Respiratory complications	–	–	–	–	–	–	+	–	–	–	–	1/11 (9)	0/10 (0)
Other	–	–	T	–	–	–	S	E	H	–	–		

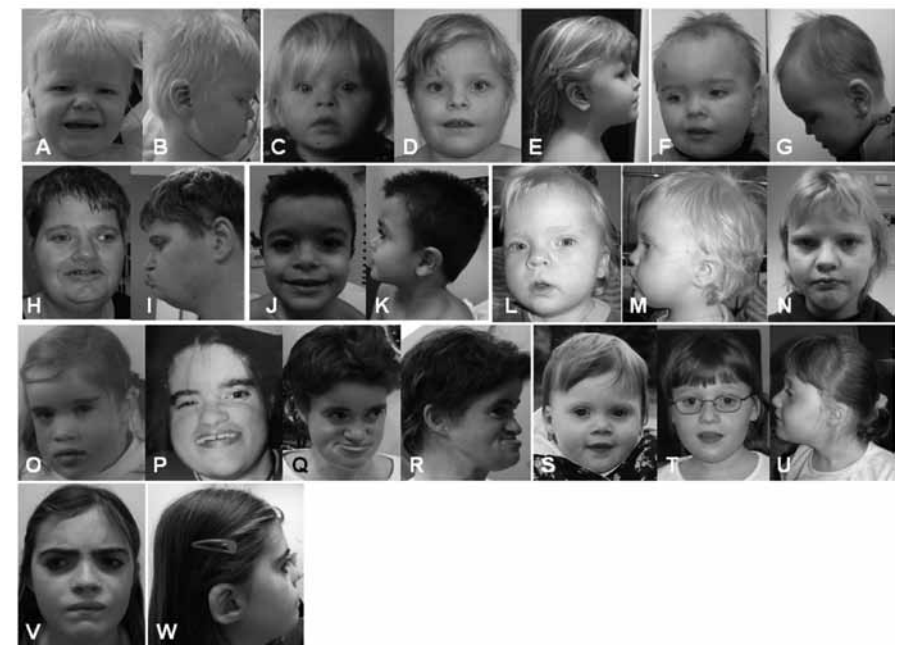
DD/ID, developmental delay/intellectual disability: mild, mild ID; mod., moderate ID; yes, ID present, level not known; E, eye anomalies: coloboma in patient 8, severe hypermetropia in patient 16, congenital cataract in patient 23; F, female; H, hormonal: central pubertas praecox; M, male; NR, not reported; S, hyperelastic skin; SS, splice site; T, teeth anomalies; VUR, vesicoureteral reflux. +, yes; –, no.

**Figure 3 A–V** Facial profiles of patients with a 9q34.3 deletion, including patient 1 at age 2 years (**A**) and 5 years (**B**), patient 2 at age 9 months (**C**), 2 years (**D**) and 6 years (**E**), patient 4 at age 5 years (**F**, **G**), patient 5 at age 11 years (**H**, **I**), patient 6 at age 1 year (**J**) and 2 years (**K**), patient 7 at age 9 months (**L**), patient 8 at different childhood ages (**M–O**), patient 10 at young childhood age (**P**, **Q**), patient 11 at age 3 years (**R**, **S**) and 5 years (**T**, **U**) and patient 12 at age 5 years (**V**). The highly recognizable facial features comprise hypertelorism, midface hypoplasia, prognathism, prominent eyebrows, cupid bow or tented upper lip and everted lower lip.



and club feet. Renal anomalies were only twice reported in this cohort. Both patients had an *EHMT1* mutation. One was diagnosed with vesicoureteral reflux (patient 19) and the other was shown to have a hydronephrosis (patient 24). Epilepsy was present in 7 of 16 patients with a microdeletion and in 2 of 11 patients with a mutation. Behavioral problems were often reported (in 10 of 16 microdeletion patients and in 8 of 11 patients with an intragenic *EHMT1* mutation) and comprised autistic features, attention deficit problems, self mutilation, aggressive and emotional outbursts/

**Figure 4 A–W** Facial appearance of patients with an *EHMT1* mutation, including patient 17 at age 2 years (**A**, **B**), patient 18 at age 1 year (**C**) and 6 years (**D**, **E**), patient 19 at age 3 years (**F**, **G**), patient 20 at age 41 years (**H**, **I**), patient 21 at age 5 years (**J**, **K**), patient 24 at age 2 years (**L**, **M**) and 10 years (**N**), patient 25 at childhood age (**O**), during teenage (**P**), and age 32 years (**Q**, **R**), patient 26 at age 2 years (**S**) and age 10 years (**T**, **U**), patient 27 at age 11 years (**V**, **W**). Facial characteristics are similar to Kleefstra syndrome patients with a 9q34.3 deletion.



crises and severe sleep disturbance. In 2 of the 5 patients who had reached adult age at the time of the examination, a behavioral change at adult age was reported. They showed alternating periods with apathy and catatonia-like behaviors with a general decline in functioning (patient 20 and 25). These neuropsychiatric conditions were recently described in detail by Verhoeven *et al.*<sup>22</sup> In 10 of the 16 patients with a microdeletion and in 10 of the 11 patients with an intragenic *EHMT1* aberration, cerebral imaging by MRI had been performed. Variable anomalies were reported in 5 and 7 patients, respectively, and included dilated ventricles, white matter anomalies, corpus callosum hypoplasia and cerebellar hypoplasia.

**Table 4** Frequency of features in presently and previously reported patients with KS

Clinical features of total number of KS cases	Mutation	
	9q34 deletions n (%)	EHMT1 mutations n (%)
Age range, years (y, mo)	0–59	2.5–41
High birth weight (>P90)	6/66 (9)	4/19 (21)
Microcephaly	32/64 (50)	4/21 (19)
Short stature	18/56 (32)	3/18 (17)
Overweight (BMI >25)	18/64 (28)	8/19 (42)
DD/ID	91/91 (100)	21/21 (100)
Heart defect	27/66 (41)	9/21 (43)
Genital anomaly (in males)	17/53 M (32)	3/7 M (43)
Renal anomaly	7/59 (12)	3/21 (14)
Recurrent infections	9/35 (26)	7/11 (64)
Hearing deficit	12/52 (23)	5/21 (24)
Gastro-esophageal reflux	10/52 (19)	3/21 (14)
Epilepsy	21/59 (36)	5/21 (24)
Behavioral/psychiatric problems	32/59 (54)	15/20 (75)
Anomalies on brain imaging	25/43 (58)	12/19 (63)
Tracheomalacia	6/53 (11)	1/21 (5)
Umbilical/inguinal hernia	9/54 (17)	2/21 (10)
Anal atresia	2/67 (3)	1/21 (5)
Musculoskeletal anomaly	13/51 (25)	4/21 (19)
Respiratory complications	8/58 (14)	1/21 (5)

Study group included 91 patients with a 9q34.3 deletion (75 previous and 16 present) and 21 patients with an *EHMT1* mutation (10 previous and 11 present). Two patients with an *EHMT1* mutation are not included, because clinical data were not available.

Tables 2 and 3 also include a summary of the clinical features reported in previous reports. Table 4 summarizes the frequency of clinical features in the total (present and previously reported patients) cohort of patients with KS, including 91 patients with a 9q34.3 deletion and 21 patients with an *EHMT1* mutation.

## Discussion

In this study 29 novel identified patients with KS are described (clinical data were not available for patients 28 and 29) and compared to 85 previously reported patients. The majority of the deletions described here (11/16) were identified by genome-wide array analysis. A large number of previous deletions were reported before genome-wide array platforms became common use in routine diagnostic settings, and were either detected by routine FISH or MLPA.<sup>3-5, 7, 9</sup> Some studies reported fine-mapping of deletions with additional specific 9q probes.<sup>6, 8</sup> Only 2 previously reported deletions were cytogenetically visible, indicating that large cytogenetically visible deletions are a relatively rare cause of KS.<sup>1, 2</sup> More recent reports also included 9q34.3 deletions identified and delineated by genome-wide array analysis.<sup>11, 19</sup> Genome-wide array analysis generally enables a more precise delineation of the breakpoints, though many array platforms have poor coverage of the 9q subtelomeric region. The use of different methods for molecular characterization in previous and present studies makes exact genotype-phenotype comparison more difficult. In agreement with the more recent reports, the sizes and breakpoints of the present deletions are heterogeneous.<sup>11, 19</sup> Previously, it was indicated that patients with microdeletions smaller than 3 Mb in size show highly similar clinical findings. In the present study we observed a tendency to a more severe ID in patients with deletions of >1 Mb in size. Three to seven patients had a deletion of more than 1 Mb (patients 4, 5 and 8; in patients 1, 9, 10 and 16 deletion size was not well defined, but possibly more than 1 Mb). In 6 of these 7 patients a severe level of ID was documented. In 9 patients a deletion smaller than 1 Mb was identified. Three of them had mild ID and in 2 patients severe ID was reported. However, the reported level of ID has to be considered with caution, because we noticed more often that at very young age the severity of the delay is sometimes underestimated and tends to become more obvious at school age when a higher level of performance is expected. In this cohort 5 out of 16 patients with a 9q34.3 microdeletion were diagnosed with a structural heart defect. Two of them had a deletion of more than 1 Mb in size. The deletion of patient 9 with a heart defect was delineated poorly and is possibly more than 1 Mb in size. The deletion of patient 15 was relatively small (270 kb). She had a perimembraneous ventricle septal defect for which an operation was not necessary. These numbers are too low to conclude that the more severe structural cardiac defects are more common in deletions sized >1 Mb. Furthermore, structural heart defects are equally present in patients with intragenic *EHMT1* mutations. Of note, in patient 4 the deletion encompasses both *COL5A1*, associated with Ehlers-Danlos type I [OMIM 130000], and *NOTCH1*, shown to be associated with aortic valve anomalies [OMIM 109730].<sup>23</sup> Therefore, the heterozygous deletion of both *COL5A1* and *NOTCH1* likely has contributed to

the cardiac valve phenotype in this patient presenting with aortic and pulmonary valve defects in addition to cardiac septal defects. Deletion of *COL5A1* likely contributed to the generalized joint hypermobility that was observed in patient 4. However, in patient 8, who also has a deletion comprising these genes, neither a heart defect nor signs of connective tissue disease were observed, so penetrance seems incomplete. Of note, 13 of the 16 identified deletions comprised the gene *CACNA1B* as well. We did not observe differences in phenotype between patients with a deletion comprising *CACNA1B* (patients 2--12, 14 and 16) and patients with a deletion excluding *CACNA1B* (patients 1, 13 and 15). *CACNA1B* is a voltage-dependent calcium channel and despite the demonstrated importance of the  $\alpha 1B$  subunit for neurotransmission in most neuronal cells during embryogenesis and after birth, the null-mutant mice showed no gross abnormalities and normal motor coordination. These data suggest that the  $\alpha 1B$  gene is not essential for synaptogenesis and neuronal differentiation during embryonic and postnatal development.<sup>24</sup> This might underscore why a heterozygous loss of this gene does not contribute significantly to the observed phenotype in 9q34.3 deletions. Moreover, Yatsenko et al.<sup>11</sup> described a familial case with a heterozygous loss of *CACNA1B* but not *EHMT1*. Affected family members had a normal development and did not have the KS phenotype. Previous reports suggested a higher prevalence of respiratory complications in patients with deletions  $\geq 3$  Mb.<sup>18</sup> In the present study respiratory complications were present in patient 4 with a large deletion of 3.85 Mb, in patient 10 with a poorly defined deletion, but possibly larger than 1 Mb in size, and in patient 7 with a 700 kb deletion. However, the respiratory problems in these patients were of different origin. Patient 4 suffered from central apneas and patient 7 and 10 had respiratory problems secondary to a viral bronchiolitis and aspiration, respectively. Central apneas have been reported in 1 previously published patient who had a cytogenetically visible deletion.<sup>9</sup> From a personal communication we know an additional patient with central apneas who has a 9q subtelomeric deletion of more than 3 Mb as well. So, it might be that central apneas are associated with deletions over 3 Mb.

Since the identification of *EHMT1* in 2005 as the gene responsible for the phenotype<sup>13, 25</sup>, mutations in *EHMT1* are increasingly being detected. So far, 23 intragenic *EHMT1* defects have been reported (including this report). Except for one (c.1858C>T), all present mutations were unique and not reported before. Presence of distinct clinical features is comparable between the present and previously reported cohorts. Mutations are scattered throughout the gene, but are more frequent on the 3' end of the gene and occur mostly in the PreSET domain (fig. 2). All mutations in this cohort, except for 1 missense mutation and 1 splice donor site mutation, are truncating mutations suggesting loss-of-function. We did not observe a correlation between the severity of the phenotype, indicated by the degree of ID

and presence of other major medical problems, and the respective domain localizations and type of the mutations.

Comparison of observed clinical features between the total (including present and previous patients) 9q34.3 deletion group and the total intragenic *EHMT1* mutation group revealed only a few remarkable differences between both groups (table 4). This is in agreement with previous observations that *EHMT1* is responsible for the core phenotype of KS and most of other associated features. *EHMT1* is an epigenetic regulator that affects gene transcription by histon modification. It is one of a rapidly growing list of ID genes that are implicated in chromatin remodeling.<sup>14, 26, 27</sup> It is likely that other associated features besides ID can be explained by the effect of *EHMT1* mutations on the expression of a variety of target genes.

In the present mutation group the mean age is higher than in the deletion group. There are no indications that patients with a deletion die at a younger adult age than patients with a mutation. There is only 1 death (cause unknown) at adult age reported. This concerns a 21-year-old male patient with a deletion.<sup>19</sup> The increased average age in the present mutation group compared to the present deletion group might be due to an older age of diagnosis in the mutation group, because deletions can be detected by standard genome-wide array analysis and thus are more easily recognized than mutations for which analysis has to be specifically requested on the base of the clinical phenotype.

Microcephaly and short stature were more frequent in the 9q34.3 deletion group (50% vs. 19% and 32% vs. 17%, respectively). Respiratory complications and tracheomalacia were also more frequently observed in the 9q34.3 deletion group (14% vs. 5% and 11% vs. 5%, respectively). Features that were observed more often in the *EHMT1* mutation group included high birth weight (9% in the deletion group vs. 21% in the mutation group) and overweight (28% and 42%, respectively). Recurrent infections and behavioral problems were also more often reported in patients with an *EHMT1* mutation (in 26% vs. 64% and in 54% vs. 75%, respectively). These observations are highly consistent with our report in 2009.<sup>19</sup> The explanation for the observed differences in frequency of certain features is unclear, but might be partly explained by inconsistent report of some features in previous studies among 9q34.3 microdeletion patients and relatively low numbers of patients with an *EHMT1* mutation which influences the figures. The distinct pattern of behavior problems observed in several teenage and adult patients with KS has recently gained more attention<sup>15, 19, 22</sup> and thus behavior problems are likely more consistently reported in recent reports.

The present report confirms to a large extent the phenotypic spectrum described in earlier reports. Most frequently observed clinical features in KS apart from the core phenotype included behavioral problems (~50--75%), various brain anomalies (~60%), heart defects (~40--45%), male genital defects (~30--40%), microcephaly



(~20--50%) and overweight (~30--40%). Interestingly, cardiac defects that we observed in this cohort included structural anomalies but also other defects. In patient 14 an aberrant muscle bundle in the left ventricle was observed. Patient 20 and 1 of the previously reported patients with an intragenic *EHMT1* mutation were both diagnosed with cardiac arrhythmias, including atrial flutter (personal communication). Epilepsy (~25--35%), musculoskeletal anomalies (~20--25%), recurrent infections (>25%), hearing problems (20--25%) and constipation (personal observation) are quite common as well. Features that are observed more rarely are renal anomalies and other congenital anomalies, gastro-esophageal reflux and respiratory complications (table 4). As concluded previously, only minor indications for phenotype-genotype correlations could be observed within the group of deletion patients (between larger (>1 Mb) and smaller deletions), as well as between the 9q34 deletion and *EHMT1* mutation group. Based on the phenotypic data presented here, we recommend cardiac screening during follow-up of KS patients, because of the possible occurrence of cardiac arrhythmias, even if no structural heart defect is present. In addition, clinicians and caretakers should be aware of the regressive behavioral phenotype that might develop at adolescent/adult age and seems to have no clear neurological substrate, but is rather a so far unexplained neuropsychiatric feature.

Identification of increasing numbers of patients with (smaller) deletions and *EHMT1* mutations will probably further increase our knowledge of the phenotypic and genotypic spectrum of KS. Furthermore, application of novel sequence technologies might lead to the identification of other genes involved in phenotypes compatible with KS. Interestingly, our group recently published that the learning and memory defects in *EHMT* mutant *Drosophila melanogaster* could be rescued when *EHMT* expression was restored.<sup>26</sup> These studies indicate that learning and memory are dynamic processes that might be rescued at least to some extent even when caused by genetic defects such as mutated *EHMT*. This opens the window in understanding the mechanisms of ID and strongly emphasizes the need for further investigations on possible intervention strategies that might become available in future for some aspects of cognitive processes.

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# 4.1.2

## **Familial Kleefstra syndrome due to maternal somatic mosaicism for interstitial 9q34.3 microdeletions**

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## Abstract

The Kleefstra syndrome (Online Mendelian Inheritance in Man 607001) is caused by a submicroscopic 9q34.3 deletion or by intragenic *euchromatin histone methyl transferase 1 (EHMT1)* mutations. So far only *de novo* occurrence of mutations has been reported, whereas 9q34.3 deletions can be either *de novo* or caused by complex chromosomal rearrangements or translocations. Here we give the first descriptions of affected parent-to-child transmission of Kleefstra syndrome caused by small interstitial deletions, approximately 200 kb, involving part of the *EHMT1* gene. Additional genome wide array studies in the parents showed the presence of similar deletions in both mothers who only had mild learning difficulties and minor facial characteristics suggesting either variable clinical expression or somatic mosaicism for these deletions. Further studies showed only one of the maternal deletions resulted in significantly quantitative differences in signal intensity on the array between the mother and her child. But by investigating different tissues with additional FISH and MLPA analyses, we confirmed somatic mosaicism in both mothers. Careful clinical and cytogenetic assessments of parents of an affected proband with an (interstitial) 9q34.3 microdeletion are merited for accurate estimation of recurrence risk.

## Introduction

The Kleefstra syndrome, previously known as the 9q Subtelomeric Deletion Syndrome (9qSTDS), is one of the first clinically recognizable subtelomeric deletion syndromes.<sup>1, 2</sup> Affected individuals have severe hypotonia with speech and gross motor delay. The facial features comprise micro- or brachycephaly, hypertelorism, synophrys, arched eyebrows, midface hypoplasia, a short nose with upturned nares, a protruding tongue, everted lower lip and down-turned corners of the mouth. Approximately half of affected individuals have congenital heart defects and 10-20% have a combination of epilepsy, behaviour and sleep disturbances. A variety of other major and minor anomalies have been reported.<sup>1-6</sup> We previously demonstrated that the syndrome is caused by haploinsufficiency of *euchromatin histone methyl transferase 1 (EHMT1)*, a gene involved in histone methylation.<sup>3, 7, 8</sup> We and others found no evidence for phenotype-genotype correlations.<sup>3, 4</sup> All individuals with Kleefstra syndrome identified so far have been single cases or sib pairs, the latter affected as a consequence of a balanced chromosomal rearrangement involving the 9q34.3 region in one parent.

Here, we report the clinical and molecular cytogenetic findings in two unrelated families where a small interstitial 9q34.3 deletion is segregating. This new observation is relevant for accurate genetic counseling in families with a proband who carries a 9q34.3 deletion.

## Case reports

### Family 1

The family is shown in Figure 1. The proband was born at term to a gravida 1 para 0 mother who had had one early miscarriage. He weighed 3,520 grams (50<sup>th</sup> centile). Glandular hypospadias was noted at birth but he was otherwise thought to be well. By the age of 7 weeks reduced muscle tone and persistent head lag were evident. At 16 weeks of age delayed motor development and lack of visual interest in surroundings were noted. By 6 months of age, axial tone had improved with better head control, but the occipito-frontal circumference (OFC) had fallen from the 25<sup>th</sup> centile at age 6 weeks to the 2<sup>nd</sup> centile by age 30 weeks. Right inguinal hernia was noted in addition to the hypospadias and both were repaired by surgery. Bilateral forefoot adduction deformities were treated with splints. By age one year, unusual short absences with hypotonia and atypical spasms were noted but EEG examination was normal as were blood tests including full blood count, urea and electrolytes, liver function tests, creatine kinase and uric acid. Cerebrospinal fluid biochemistry including protein, glucose and lactate levels, was also normal. Global



**Figure 1** Photograph of family 1. The proband (left) with his younger brother and his mother. Note the characteristic facial appearance of Kleefstra syndrome in the proband and the mild facial features in the mother. The younger brother shows marked hypotonia.



delays in development became more evident, with sitting at 1 year, walking at 2.5 years and emergence of single words at 3 years. A Giemsa banded karyotype revealed a balanced, reciprocal translocation (46,XY,t(2;13)(q31;q34). This balanced reciprocal translocation was inherited from the proband's healthy father and was judged not to be of any significance in relation to the proband's condition. There was no evidence of a fragile X syndrome triplet repeat expansion or a chromosome 15 DNA methylation abnormality that is associated with Angelman syndrome.

At the age of 21 months, a cerebral MRI was performed which showed white matter abnormalities that were interpreted as T2 hyperintensities in the peritrigonal regions, suggestive of periventricular leucomalacia.

In estimating the chance of recurrence in a sibling of what was then an undiagnosed disorder in the proband, the family history was considered pertinent because mother had had some learning difficulties and had required additional help at school. No other health problems were admitted. Mother's OFC was just under the 50<sup>th</sup> centile. The possibility that the proband had a sex-linked disorder was

considered (HbH inclusions indicative of ATRX syndrome were sought but not found). The parents were advised that in the absence of a definite diagnosis, there was a moderate chance of recurrence of unexplained learning difficulties in a second child.

Some years later, after a second early miscarriage, the parents' second son presented to the paediatric service at the age of 9 weeks following concerns about his neurodevelopmental process. He had been born by emergency caesarean section at 37 weeks following an uneventful pregnancy, his birth weight was close to the 50<sup>th</sup> centile (3,400 grams) and his OFC was on the 10<sup>th</sup> centile. There were no perinatal problems, but axial hypotonia and post axial polydactyly affecting one foot were noted in the newborn period. These observations caused concern that the baby had the same undiagnosed, neurodevelopmental condition as his older brother. At the age of 9 weeks he attended the paediatric service with axial hypotonia and marked head lag, visual inattention and he was not smiling. He had increased tone of the upper limbs with fisting and brisk peripheral reflexes. He was reported to be feeding poorly. His weight was 4,800 grams (25<sup>th</sup> centile) and his OFC had fallen to the 2<sup>nd</sup> centile. He had unusual morphology of the ear lobes. Subsequent neurodevelopmental progress was similar to the proband and was abnormal in all domains. In particular, he had difficulties with feeding, partly caused by tongue protrusion. He had abnormal speech and language development with babble but no single words and no symbolic play at the age of 2 years. At this age he was, however, sitting independently and would pull to stand and attempt to cruise around the furniture. Like his brother, he had had problems with vomiting and poor weight gain as well as a poor sleep pattern. Also similar to his brother, his parents reported short or generalised clonic events consistent with epileptic seizures but associated with normal EEG examinations. His OFC continued to grow along the 2<sup>nd</sup> centile.

## Family 2

This family is shown in figure 2. The proband is the second child of consanguineous parents. She was born at a gestational age of 36 weeks and 6 days and had a birth weight of 3,030 grams (50<sup>th</sup> centile). The pregnancy had been uneventful. She had a perinatal infection from which she recovered after antibiotic treatment. During the first months of life she was a very quiet, hypotonic baby and she showed very little development. At the age of 2 months she had pneumonia which was attributed to swallowing difficulties and gastro esophageal reflux. At that time a heart murmur was noticed but there was no evidence of a congenital heart defect at ultrasound and electrocardiography. She had mild dysmorphic features, including hypertelorism, depressed nasal bridge and an upturned nose (Fig. 1 A). Routine karyotyping showed a normal female chromosomal pattern (46,XX).



**Figure 2** Photographs of family 2. The proband at the age of 2 months (**a**), 3 years (**b**), 4 years and 6 months (**c, d**) and 5 years (**e**), and her mother (**f, g**). Note the hypertelorism, broad and depressed bridge of the nose, flared nostrils (**a, b, d, e**), mild midface hypoplasia (**c**) and broad mouth with full lips in the proband (**b, d, e**). The mother has marked obesity and minor dysmorphic features, including a hypoplastic midface, small palpebral fissures, a depressed nasal bridge and anteverted nares (**f, g**).



A brain MRI at the age of 18 months did not show any abnormalities. Hearing was not impaired and X-rays of femur and calcaneus revealed no stippling indicative of peroxisomal disorders and metabolic screening of plasma and urine did not show any abnormalities.

Upon evaluation at the age of 3 years she did not speak and all her motor milestones were delayed. She had started rolling at the age of 11 months, had been able to sit without support since the age of 12 months and had started walking at the age of 2,5 years, but she had a very unstable gait. The parents reported her being very cheerful in general, but she did have tantrums and occasionally self-harm. Physical examination revealed a length of 94 cm (2<sup>nd</sup> centile), a weight of 15 kg (70<sup>th</sup> centile) and a head circumference of 48 cm (16<sup>th</sup> centile). She was hypotonic and had hypermobile joints. She showed stereotypic hand movements and had an unsteady gait. She had coarse facial features, hypertelorism, blue sclerae, a broad and a

depressed nasal bridge and a short nose with anteverted nares, a mildly hypoplastic midface, a wide mouth with thick lips, small teeth and a dry skin (Fig. 2 B). DNA analysis of the *MECP2* gene, MLPA MR deletion and duplication syndromes (including the 17p11.2 region for Smith Magenis syndrome)(MLPA-kit: P064, MRC-Holland), MLPA of the subtelomeric regions (MLPA-kit: P036C and P070, MRC-Holland) and analysis of the methylation pattern of the Prader-Willi Angelman region (15q12) did not show any abnormalities.

At 5 years of age, when she was reevaluated, she spoke two-word sentences, had a steadier gait and showed less tantrums. She seemed to have a high pain threshold. There were no signs of epilepsy, hearing problems or visual problems. Physical examination revealed dysmorphic features in conformity with those observed at the age of 3 years, except for an asymmetry of the palpebral fissures, with mild ptosis on the left side (Fig. 2 E).

The family history revealed that the proband's older sister, who has had normal psychomotor development, had a combined immune deficiency of unknown cause, for which she had a bone marrow transplantation. The girls' mother experienced learning difficulties and she did not finish high school. She suffered from a depression and was quite shy. Physical examination revealed obesity and minor dysmorphic features, including a hypoplastic midface, small upslanting palpebral fissures, depressed nasal root and anteverted nares (Fig. 2 F-G).

## Methods

### MLPA

Routine MLPA studies to search for subtelomeric abnormalities were performed using the P070 kit of MRC-Holland as previously described.<sup>9</sup>

### Fluorescent in situ hybridization (FISH)

FISH analysis on slides prepared from buccal swabs was performed using directly labeled BAC probes RP11-417A4 (Chr9: 139,523,178 – 139,716,008 Mb (hg18)) and RP11-467E5 (Chr9: 139,633,667 - 139,799,808 Mb (hg18)) (BlueGnome Ltd, Cambridge, UK) in combination with the centromere probe for chromosome 9 (Vysis Inc., Downers Grove, IL, USA). FISH was performed according to the manufacturer's instructions and our established laboratory protocol.

### Genome wide array analysis

In family 1 the Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA, USA) was used to screen for CNVs. Genome-wide SNP-array hybridization was performed following the manufacturer's instructions. Genome wide copy number

analysis was performed using the Affymetrix Genotyping Console version 4.0 and the Affymetrix Genotyping Console Browser version 1.10.12, and a reference set of 47 in-house control DNA samples.

In family 2 the Agilent 105k array (Agilent Technologies, Palo Alto, CA) has been used to screen for CNVs and to determine the 9q subtelomeric deletion. Labeling and hybridization procedures were performed according to the manufacturer's instructions. Images of the arrays were obtained using a microarray scanner G2505B (Agilent technologies) and image analysis was performed using feature extraction software (version 9.1; Agilent Technologies). The Agilent CGH-v4\_91 protocol was applied using default settings. Data analysis was performed with Agilent analysis software (DNA analytics, Agilent Technologies, Inc.). The predicted copy numbers of each CNV were determined using the Hidden Markov Model (HMM) incorporated in each data analysis software package.

Results

Family 1

Routine subtelomeric MLPA studies in the proband revealed a decreased intensity of the 9q probe located in *EHMT1*, indicative for a deletion of this region. Further segregation studies by MLPA in the younger brother and the mother, revealed a similarly decreased signal of the *EHMT1* probe in both. The maternal grandparents of the proband were not available to be examined.

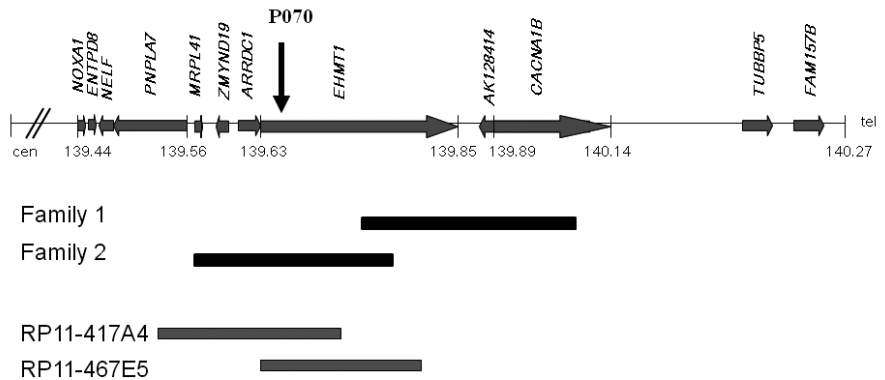
We confirmed the findings by analyzing DNA material of the proband and his mother on the 6.0 SNP array showing an identical loss of 233 kb in chromosomal band 9q34.3 (Fig. 3) between markers SNP\_A-2178662 (139,747,286) and CN\_1303976 (139,979,603) (UCSC browser, March 2006, NCBI Build 36.1/hg18; <http://genome.ucsc.edu>), with a proximal breakpoint between exons 4 and 5 of the *EHMT1* and a distal breakpoint between exons 10 and 11 of the *CACNA1B* gene. Similar signal intensities were observed in the proband and his mother, so our 6.0 SNP array gave no indications for a mosaic pattern in the mother.

No additional CNVs were detected, neither in the proband, nor in his mother.

Family 2

Genome wide array-CGH in the proband revealed an approximately 200 kb deletion in chromosome band 9q34.3 (fig 3). The start of the deletion is between base pairs 139,567,629 (probe A\_14\_P101384) and 139,578,983 (probe A\_14\_P127304) and the end is between base pairs 139,757,789 (probe A\_16\_P02195928) and 139,777,287 (probe A\_14\_P124669), indicating a minimal deletion of 179 and a maximal deletion of 210 kb which at least contains the first five exons of the *EHMT1* gene. (UCSC

**Figure 3** Schematic overview of the deleted regions in chromosome band 9q34.3 in probands and mothers of family 1 (139,747,286-139,979,603 Mb) and 2 (139,757,789-139,777,287 Mb) delineated by genome wide array analysis.



Relative positions of *EHMT1*, flanking genes, the MLPA probe of MLPA kit P070 (MRC-Holland) and the FISH probes in this region are indicated. Both deletions involve *EHMT1*.

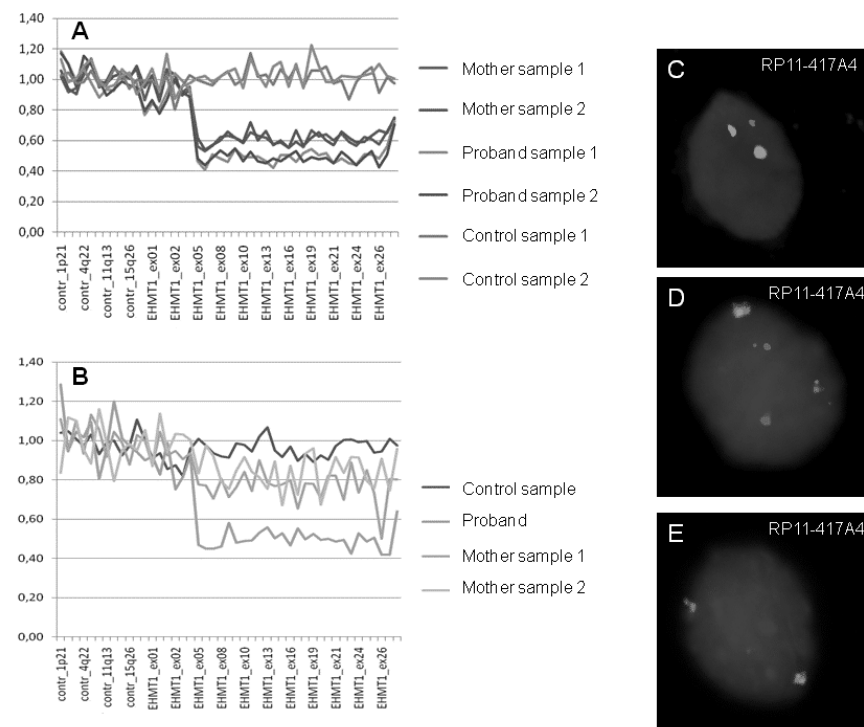
browser, March 2006, NCBI Build 36.1/hg18; <http://genome.ucsc.edu>). The deletion was confirmed with FISH using BAC probes RP11-417A4 and RP11- 467E5. Array CGH testing of the parents showed that the patient's deletion was inherited from her mother, with the mother showing a less pronounced loss as determined by the log ratio (not shown here), suggestive of mosaicism.

Mosaic pattern detection

To further investigate whether the milder phenotypes in the mothers might be explained by presence of the 9q deletion in a mosaic pattern, we performed additional FISH and MLPA experiments in different cell types.

In family 1 MLPA analysis using probes in exons 1, 2, 5, 8, 10, 13, 16, 19, 21, 24 and 26 of the *EHMT1* gene was performed in blood lymphocytes and fibroblasts, each on two different samples. This revealed in both cell types a mosaic pattern in the mother, showing a lower percentage of cells with the 9q deletion in the fibroblasts than in the lymphocytes. (Fig.4 A and B)

**Figure 4** Mosaic pattern detection in family 1 and 2. The results for 4 control probes and exons 1, 2, 5, 8, 10, 13, 16, 19, 21, 24 and 26 of the *EHMT1* gene are indicated. (a) and (b) indicate the results of the MLPA analysis in the proband and the mother of family 1, showing mosaicism for the 9q34.3 deletion in both blood lymphocytes (a) and fibroblasts (b) of the mother. Note that a lower percentage of the fibroblasts carries the 9q deletion as compared to the lymphocytes. (c)-(e) show the results of the FISH analysis on cells derived from buccal swabs in the proband and the mother of family 2. (c) shows only one red signal, indicating the deletion of probe RP11-417A4 (red color) in chromosome region 9q34.3 in the proband. (d) and (e) show mosaicism in the mother with a normal signal pattern for probe RP11-417A4 in (d) (two red signals) and only one red signal in (e), indicative for a loss in 9q34.4 in this cell. The centromere 9 probe is labeled green and shows a normal FISH pattern (two signals) in (c)-(e).



In family 2, interphase FISH analysis on a buccal swab sample from the mother showed the deletion in 80% of her cells (50 cells analyzed) indicating that she is a mosaic carrier of the 9q34.3 deletion (Fig. 4 D-E). This argues for a *de novo* event in the mother.

## Discussion

This is the first report of familial Kleefstra syndrome. In two different families three cases of Kleefstra syndrome were identified and explained by mosaicism in their mothers for a 9q.34.3 deletion encompassing part of the *EHMT1* gene. Importantly, both mothers showed a significantly milder phenotype. Thus, in family 1 a sex-linked, Angelman-like disorder was first suspected and in Family 2 the *MECP2* (Rett syndrome) gene was analysed before Kleefstra syndrome was correctly diagnosed in the proband.

Familial transmission of autosome imbalances with milder phenotypes that display variable expression, such as deletions in 22q11.2 and duplications in 15q11q13, are reported<sup>10-23</sup> and Barber has listed 200 families with a directly transmitted, cytogenetically visible, autosomal anomaly.<sup>24</sup> Most familial cases are explained by either a parental balanced chromosomal rearrangement or parental mosaicism. Mosaicism can either be germline or somatic. Carriers of a somatic mosaic imbalance can be similarly affected, less affected, or unaffected (with preservation of reproductive fitness). There are literature reports of somatic mosaicism for both the severe and less severe autosome imbalance syndromes including mosaic 17p11.2 del (Smith-Magenis syndrome), mosaic 22q11.2 del (DiGeorge/VCF syndrome), mosaic 3q29 del, mosaic 22q13 del, mosaic 20p del (Alagille syndrome), mosaic 8q24 del (Langer Giedion syndrome), mosaic 5pdel (Cri-du-Chat syndrome) and mosaic 4qter del.<sup>25-32</sup> But in both families with Kleefstra syndrome whom we report, it was a surprise to discover the proband's condition was transmitted by a mildly affected, mosaic mother.

Somatic mosaicism for chromosomal imbalances may be more frequent than appreciated due to inability of diagnostic tests to detect low level mosaicism that causes mild or even no clinical signs. In conventional cytogenetic analysis, low level mosaicism may be missed unless large numbers of cells are tested and even then, the cell culture process may complicate detection due to selective disadvantage of cells with the chromosomal imbalance.<sup>33</sup> Genome-wide array analysis was shown to be capable of consistently detecting mosaicism with a level as low as 20%.<sup>33</sup> Consequently, the introduction of genome wide array technologies in routine diagnostic settings is likely to increase the detection rate of mosaic

chromosomal imbalances. Noteworthy, in family 2 array analyses suggested the presence of a mosaicism in the mother based on a less pronounced decrease in signal intensity for the 9q34.3 region as compared to the proband, whereas in family 1, the signal intensities were similar in mother and her affected child. MLPA proved a reliable and efficient method to identify mosaicism in this latter family showing reproducible, quantitative differences in signal intensity between the mother and her child. Moreover, these analyses permitted detection of different percentages of mosaicism in peripheral blood cells compared to fibroblasts (Fig. 4 A and B).

Chromosomal imbalances can arise both meiotically and somatically.<sup>34</sup> Most *recurrent* interstitial chromosomal imbalances result from meiotic non allelic homologous recombination (NAHR) within recombination hotspots. Examples are the 1.4 Mb type 1 deletion in neurofibromatosis 1 and the 3.7 Mb deletion in 17p11.2 causing Smith-Magenis syndrome.<sup>35,36</sup> In these specific deletions, the chance of parental somatic mosaicism might be considered very low because of the presumed meiotic origin of the imbalance. Interestingly, NAHR was also shown to play a significant role in *non-recurring* chromosomal rearrangements and can comprise either inter-or intrachromosomal unequal crossing over events.<sup>36,37</sup> It appears that somatic NAHR is mediated by intrachromosomal exchange during mitosis and germline NAHR by interchromosomal non-homologous pairing during meiosis.<sup>35</sup> Furthermore, NAHR is frequently involved in the generation of genomic rearrangements in somatic cells.<sup>34,38</sup> This suggests that somatic NAHR might be one explanation for the occurrence of mosaic chromosomal imbalances.

In conclusion, this report emphasizes that parental somatic mosaicism can occur in a parent of a child with a severe chromosomal imbalance syndrome such as Kleefstra syndrome and mild learning disabilities may be the only clue. Detailed chromosome studies that may extend to array CGH, FISH or MLPA experiments in different tissues are required to demonstrate somatic mosaicism and alert the parents to the high risk of syndrome recurrence.

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# 4.2

## Adult phenotypes in Angelman- and Rett-like syndromes

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## Abstract

Angelman- and Rett-like syndromes share a range of clinical characteristics, including intellectual disability (ID) with or without regression, epilepsy, infantile encephalopathy, postnatal microcephaly, features of autism spectrum disorder and variable other neurological symptoms. The phenotypic spectrum generally has been well studied in children, however evolution of the phenotypic spectrum into adulthood has been documented less extensively. To obtain more insight in natural course and prognosis of these syndromes with respect to developmental, medical and socio-behavioural outcomes, we studied the phenotypes of 9 adult patients who were recently diagnosed with 6 different Angelman- and Rett-like syndromes. All these patients were ascertained during an ongoing cohort study involving a systematic clinical genetic diagnostic evaluation of 250 mainly adult patients with ID of unknown etiology. We describe the evolution of the phenotype in adults with *EHMT1*, *TCF4*, *MECP2*, *CDKL5* and *SCN1A* mutations, and 22qter deletions, and also provide an overview of previously published adult cases with similar diagnoses. These data are highly valuable in adequate management and follow-up of patients with Angelman- and Rett-like syndromes and accurate counseling of their family members. Furthermore they will contribute to recognition of these syndromes in previously undiagnosed adult patients.

## Introduction

Clinical characteristics of known syndromes including associated health and behaviour issues and recognizable facial features are generally well studied and described in patients at childhood. Often little is known about evolution of the phenotype across life span. Information on health and social outcomes is necessary for an adequate management and follow-up of patients with specific intellectual disability (ID) syndromes and enables careful counseling of family members regarding prognosis, natural course of the disease and life expectancy. Delineation of phenotypes at adult age will also contribute to recognition and diagnosis of ID syndromes at adult age.

In the past decade improvements in diagnostic and research technologies have led to the identification of several novel chromosomal microdeletions and -duplications as well as single genes that are associated with known and novel ID syndromes, referred to as "Angelman- and Rett-like syndromes."<sup>1-11</sup> After the initial identification of the gene *methyl CpG binding protein 2 (MECP2)* as the cause of Rett syndrome<sup>2</sup>, additional genes associated with a Rett-like/ aspecific Rett syndrome phenotype have been subsequently identified. These include *forkhead box G1 (FOXP1)*, *cyclin-dependent kinase-like 5 (CDKL5)* and *myocyte enhancer factor 2C (MEF2C)*.<sup>1, 6-7, 11</sup> Furthermore, in several patients with a clinical diagnosis of Angelman-like syndrome, mutations in *MECP2* and *CDKL5* have been identified.<sup>12-15</sup> In addition several mutations in the gene *transcription factor 4 (TCF4, Pitt Hopkins syndrome)* have been identified in patients with Angelman-like phenotypes as well.<sup>16</sup> So, Angelman- and Rett-like syndromes have an overlapping phenotypic spectrum with heterogeneous underlying genetic defects which can mimic each other. The phenotypic spectrum comprises (severe) ID with or without regression, epilepsy, infantile encephalopathy, features of autism spectrum disorder, stereotypies and hand apraxia, postnatal microcephaly, autonomic dysfunction (including breathing anomalies, vasomotor disturbance and gastro-intestinal symptoms), and other neurological symptoms, including ataxia and spasticity. Furthermore, various underlying genetic defects are thought to act in similar and/or interacting pathways. For example, *MECP2* is thought to be involved in the epigenetic regulation of *UBE3A* (associated with Angelman syndrome).<sup>17-18</sup> Also, *MECP2*, *CDKL5* and *MEF2C* were shown to interact directly which suggest involvement in a common pathway.<sup>11, 19</sup>

During an ongoing cohort study among mainly adult patients with so far unexplained ID, we identified in 9 adult patients with Angelman- and Rett-like phenotypes an associated genetic defect. We found mutations in the genes *TCF4*, *CDKL5*, *euchromatic histone-lysine N-methyltransferase 1 (EHMT1)*, *MECP2* and *sodium channel, voltage-gated, type I, alpha subunit (SCN1A)*, and small terminal deletions in chromosomal region 22q13.3, associated with Phelan- McDermid syndrome. Here we describe the evolution of their phenotypes up to different adult ages and give an overview of previously reported adult cases.

## Patients and methods

All patients were ascertained during an ongoing cohort study among patients with ID of unknown etiology. This study was approved by the local ethical committee. Patients were selected from a large population of approximately 4000 people living in residential settings associated with three service providers for the care of people with ID in the eastern part of the Netherlands. Patients were selected on the base of an IQ level  $\leq 70$  in combination with presence of one or more of the following features: micro-or macrocephaly; growth retardation or overgrowth; neurological features (for example ataxia, spasticity, neurodegenerative signs), congenital anomalies (i.e. congenital heart/renal/skin/genital defects); consanguinity and/or positive family history for ID; and/or dysmorphic features. More than 80% of the patients in the total cohort were adult patients. After obtaining written permission from the parents or legal representative for participation in this study, patients were invited to visit the department of human genetics at the Radboud University Nijmegen Medical Centre for an extensive multidisciplinary clinical evaluation. Specific DNA diagnostic tests were requested when indicated. In all patients genome wide SNP (Single Nucleotide Polymorphism) array analysis was performed according to the standard Affymetrix GeneChip protocol (Affymetrix, Inc., Santa Clara, CA, USA). In eight patients the Affymetrix 250k array platform was used and in one patient (patient 7) the Affymetrix 2.7 M array platform. Copy number estimates were determined using the updated version 2.0 of the CNAG (Copy Number Analyzer for Affymetrix GeneChip mapping) software package (Affymetrix 250k SNP array) or the ChAS (Chromosome Analysis Suite for Affymetrix GeneChip mapping) software package (Affymetrix 2.7M array). The normalized ratios were subsequently analyzed for genomic imbalances by a standard Hidden Marker Model (HMM). CNVs were mapped according to the UCSC genome browser build May 2004 (NCBI35/Hg17) for the Affymetrix 250k array platform and to build March 2006 (NCBI36/Hg18) for the Affymetrix 2.7M array platform.

All patients underwent screening metabolic tests in urine and serum as well, including quantification of lactate, amino acids, creatine biosynthesis, carnitines, organic acids, purines and pyrimidines, and transferrin subfractions.

### Segregation analysis

When possible, segregation of genetic/chromosomal variants was tested in the parents and/or other family members by SNP array, Multiplex Ligation-dependent Probe Amplification (MLPA), Fluorescence In Situ Hybridization (FISH) or sequencing of the involved gene.

## Results

### Patient 1: Kleefstra syndrome (*EHMT1*)

This female patient was ascertained at age 41 years. She was born at term after an uncomplicated pregnancy and delivery in breech position. Birth weight was more than 3 kg ( $>10^{\text{th}}$  centile). At the age of six weeks she was admitted to the hospital because of persistent excessive regurgitation and weight loss, probably due to severe gastro-esophageal reflux. Her motor development was mildly delayed with walking independently at age 18 months. Speech development was more severely delayed and she was not able to speak single words until the age of 3 years. Contact making was diminished and she had mood swings. An intravenous pyelogram at age 10 years, because of recurrent urinary tract infections, showed no abnormalities. Ophthalmological evaluation revealed substantial strabismus. At age 38 years her developmental age as assessed by the WISC-RN scale was 5.5 years with a discrepancy between verbal capacities (age equivalent 4.3 years) and performat capacities (age equivalent 6.6 years). She was able to walk, to talk in short simple sentences and needed some help in self care. Around age 38 years she presented with a remarkable change in her behaviour pattern, including periods of apathy, decreased emotional responsiveness, staring, low motor activity, stupor and sleep disturbance, characterized by frequent awakenings during the night and daytime sleepiness. Detailed behavioral characteristics have been published elsewhere [Verhoeven et al., 2011: patient 1]. Furthermore, impulsivity and aggressive outbursts were reported. She also showed focal myoclonic twitches of the right shoulder. Electro-encephalography was repeatedly normal. Brain MRI showed multifocal poorly defined abnormal white matter signal intensities, mainly localized deeply in the parietal lobe. In addition, bilaterally abnormal signal intensity in the globus pallidus was noticed. These abnormalities were not indicative for perinatal asphyxia. Cardiologic evaluation revealed hypertrophy of the left ventricle and paroxysmal atrial fibrillation. Upon physical examination at age 41 years her height was 176 cm ( $75^{\text{th}}$  centile), weight 103 kg ( $>98^{\text{th}}$  centile) and head circumference 53.5 cm ( $10^{\text{th}}$  centile). Facial dysmorphic features comprised a flat midface, slight upslant of the palpebral fissures, short philtrum, prognathism, and full everted lower lip (fig. 1A-B). Chromosomal analysis by 250k SNP array analysis was normal and screening metabolic tests revealed no abnormalities. Because of the clinical suspicion of Kleefstra syndrome without a deletion in chromosomal region 9q34.3, mutation analysis of *EHMT1* was performed and revealed a pathogenic mutation in the splice site of intron 20 of the *EHMT1* gene (c.3087+1G>T). Segregation analysis in the parents was not possible.



**Figure 1** Clinical pictures, **A** and **B** show the typical facial features of Kleefstra syndrome in patient 1. Note the midface hypoplasia, arched eyebrows and prognathism. **C** and **D** show the typical facial appearance of Pitt Hopkins syndrome in patient 2. Note the coarse face with prognathism, broad mouth with down turned corners and large nose. **E-H** show patient 3 with a mutation in *MECP2* (**E-F**) and his carrier sister (**G-H**). **I-J** show patient 4 in whom we identified a mutation in *CDKL5*. Note her coarse face. **K-N** show patient 5 (**K-L**) and 6 (**M-N**) with Dravet syndrome. **O-P** show patient 7 with Phelan-McDermid syndrome. Note the long face and full lips. **Q-T** show the brother pair with Phelan-McDermid syndrome. Note the long face, prominent chin and large ears in both brothers.



### Patient 2: Pitt-Hopkins syndrome (*TCF4*)

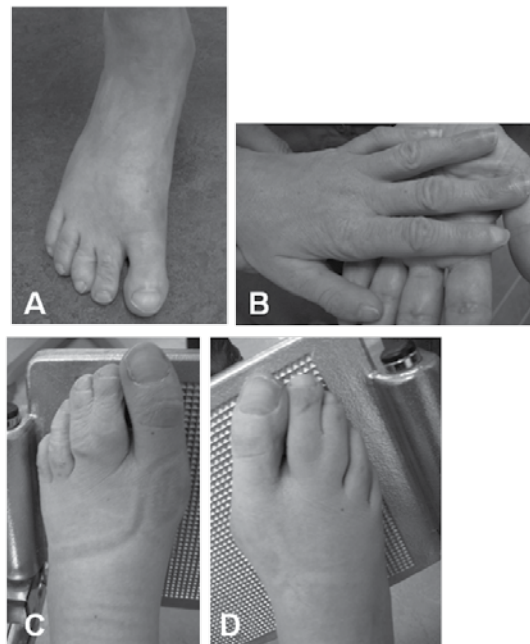
This male patient was ascertained at the age of 40 years. He was born at term after an uneventful pregnancy and delivery. Birth weight was 3200 grams (>20<sup>th</sup> centile). His psychomotor development was severely delayed. He was able to sit independently since the age of 4 years and able to walk at 8 years of age. He did not develop

speech. His behaviour was characterized by severe pica behavior, including the drift to ingest soft objectives such as socks. Several times he underwent a gastroscopy to remove socks from his stomach. To prevent further ingestion incidents, he was prescribed to wear a helmet with face mask. In addition he showed hyperactive and self mutilation behaviours and he had a high threshold of pain. During puberty he frequently had outbursts of crying, but later on he was generally in a happy mood. Apart from an atopic constitution presenting with allergy, asthma and eczema, his general health was quite good. After a fall at the age of 36 years, in addition to a fresh compression fracture of the twelfth thoracic vertebra, multiple old fractures, including metacarpal, humeral and femoral fractures, were detected and he was diagnosed with osteoporosis. Ophthalmological evaluation revealed myopia. Upon physical examination at the age of 40 years his height was 173 cm (>2<sup>nd</sup> centile), weight 56 kg (16<sup>th</sup> centile) and head circumference 53.5 cm (0.6<sup>th</sup> centile). He showed evident facial dysmorphism including remarkable prognathism, a broad nose with full tip, hyperplastic alae nasi, broad mouth with downturned corners and widely spaced teeth (fig. 1C-D). The chest was asymmetric with a mild pectus excavatum and he had a mild left convex scoliosis. His feet were flat and both hands showed short fifth fingers and tapering of the fingers with concave nails (fig. 2A-B). On the right hand a single palmar crease was present. He preferred to sit on the ground with his legs sideward rotated to the left. He had difficulties to stand up from this position and showed a mildly increased muscular tone. Chromosomal analysis by 250k SNP array analysis was normal and screening metabolic tests revealed no abnormalities. Because of some facial characteristics of Pitt Hopkins syndrome, mutation analysis of *TCF4* was requested and revealed a known pathogenic mutation (c. 1739G>A (p. Arg580Gln)). Segregation analysis in the parents showed that the mutation had occurred *de novo*.

### Patient 3: familial *MECP2* mutation

This male patient was ascertained at the age of 27 years. Pregnancy and birth were uncomplicated. Birth weight was 3750 grams (50<sup>th</sup>-75<sup>th</sup> centile). After the age of 24 months his parents noticed a delay in his psychomotor development. He was able to walk independently between age 2 and 2.5 years and could speak a few single words. At the age of about 12 years he had developed tonic clonic seizures. Seizures persisted on anti-epileptic drug treatment with a frequency of 1-2 times a day. Cerebral imaging by a CT scan at that time and later at adult age by MRI revealed no abnormalities. At adult age his total IQ level as assessed by the WISC was 33. His behaviour was characterized by lack of initiative and he was very calm, introverted and friendly. He had features of autism spectrum disorder and periodic tics. Since his early twenties he showed a progressive decline in general functioning,

**Figure 2** Clinical pictures, **A** and **B** show clubbing of toe and finger nails in patient 2 with a mutation in *TCF4*. Figures **C** and **D** show 2-3 toe syndactyly in patient 7 who was diagnosed with Phelan-McDermid syndrome.



including loss of speech (before he was able to talk in 2-3 word sentences), progressive neurological symptoms including walking difficulties with frequent falling, tremors of both arms, and apathy (showing significantly less initiative than before). He needed more help in self care as well. During the nights he frequently awakened and wandered around. After the start of anti-depressive drug treatment he seemed to improve a little, but did not regain his former level of functioning. On physical examination at the age of 27 years he had a height of 176 cm (16<sup>th</sup> centile), a weight of 51.5 kg (2<sup>nd</sup> centile) and a head circumference of 57 cm (25<sup>th</sup>-50<sup>th</sup> centile). He had a mild brachycephaly and a long face with a pronounced chin. Other dysmorphic features included a full upturned tip of the nose and a broad mouth with full lips and everted lower lip (fig.1E-F). He had narrow feet and hands with slender digits. His muscular tone was generally slightly increased and the reflexes were symmetrically brisk. There was a generalized muscular atrophy and a tremor of the arms.

The family history mentioned learning disabilities in the mother and his 3 years younger sister. The mother was admitted to a psychiatric hospital because of severe psychiatric problems. His sister had followed special education. At the age of 24 years she was employed as a packer at a distribution office and lived independently together with her husband. On physical examination she had a height of 167 cm (25<sup>th</sup> centile), a weight of 76 kg (90<sup>th</sup> centile) and a head circumference of 54.3 cm (20<sup>th</sup> centile). She had no evident dysmorphic features (fig. 1G-H).

Chromosomal analysis by 250k SNP array analysis and screening metabolic tests revealed no abnormalities. Because of the deterioration in functioning, neurological symptoms and a family history suggestive for an X-linked inheritance pattern, mutation analysis of both *ARX* and *MECP2* was performed. This revealed a pathogenic hemizygous late truncating mutation in *MECP2* (c.1233\_1243del (p.Ser411fs)). Segregation analysis showed an identical heterozygous mutation in the sister. Mother was not available for testing.

#### **Patient 4: Rett syndrome variant with infantile spasms (*CDKL5*)**

This female patient was diagnosed at age 47 years. Pregnancy and birth were uncomplicated. The first six months of her life she was doing well. At the age of six months she developed seizures and since then she had a delay in psychomotor development. Her motor development was only mildly delayed, however, she never learnt to speak. At adult age her ID was profound. The seizures persisted during her life span and despite treatment with multiple anti-epileptic drugs she suffered from frequent (atonic) seizures. In addition she developed a mild spasticity. She had a friendly personality and her behaviour was characterized by hyperactivity, self mutilation and no sense of fear. At the age of 40 years a cerebral CT-scan -which was made because of a fall from a horse- showed signs of hypoplasia of the corpus callosum. The family history documented ID due to rhesusantagonism in two sons from two different brothers from the father. Upon physical examination at the age of 47 years she had a height of 162.5 cm (<16<sup>th</sup> centile), a weight of 45 kg (2<sup>nd</sup> centile) and a head circumference of 53 cm (< 16<sup>th</sup> centile). She had prominent cheekbones, a mild prognathism, deep-set eyes and blepharochalasis. The ears seemed to be slightly high positioned and she had a full upturned nose with hypertrophic alae nasi. The lips were full and she missed several teeth due to caries (fig. 1I-J). She had thin limbs and small hands. Her feet showed slight clino- and syndactyly of the second and third toes. Chromosomal analysis by 250k SNP array analysis was normal and screening metabolic tests revealed no abnormalities. Because of the severe ID combined with persistent therapy resistant seizures and the Rett syndrome-like phenotype, mutation analysis of *MECP2*, *CDKL5*, *FOXP1* and *SCN1A* was performed and a pathogenic splice site mutation in intron 7 of *CDKL5* was found (c.464-1G>A). Segregation analysis in de parents was not performed.



## Patients 5 and 6: Dravet syndrome (*SCN1A*)

### Patient 5

This male patient was diagnosed at age 34 years. He was born at term after an uncomplicated pregnancy and birth. Birth weight was 4000 grams (<90<sup>th</sup> centile). The first 5 months of his life he was doing well. At age 5 months he developed seizures after vaccination. Since then he had regularly absences but developed normally according to his parents. At the age of two years seizure frequency increased and his speech gradually declined. At three years of age he had a status epilepticus followed by a coma state of several days. After awakening he had a severely retarded psychomotor function and furthermore a right hemiplegia and was no longer able to sit or walk. Gradually the hemiplegia partially recovered and he learnt to walk again, though he did not regain the ability to speak. The epilepsy persisted throughout his life and he suffered from intractable seizures (for which he wore a helmet), despite treatment with multiple anti-epileptic drugs. At adult age he had a profound ID (age equivalent 10-18 months). His behaviour was very friendly and characterized by little initiative, obsessive traits and a self mutilation (pulling out his nails). He had a very high threshold of pain. He recurrently had upper and lower airway infections. Upon physical examination at age 34 years his height was 193 cm (90<sup>th</sup> centile), his weight 84 kg (>50<sup>th</sup> centile) and his head circumference 59 cm (80<sup>th</sup> centile). He had a slight micrognathia and several scars on his face due to recurrent epileptic falls. The ears were large and the lips full, but his appearance was not quite dysmorphic (fig. 1K-L). He was able to walk but he was somewhat hindered in his motor function by a mild right-sided hemiplegia. Chromosomal analysis by 250k SNP array analysis was normal and screening metabolic tests revealed no abnormalities. Because of the severe ID and intractable seizures mutation analysis of *SCN1A* was performed and revealed a pathogenic mutation in exon 26 (c.5304T>G (p.Ser1768Arg)). Segregation analysis in the parents showed that the mutation had occurred *de novo*.

### Patient 6

This patient was ascertained at age 49 years. Pregnancy and birth were uneventful. He developed seizures after vaccination at about six months of age. His psychomotor development was delayed. Motor milestones were only mildly delayed according to his brother. Speech development was more severely delayed and he only learnt to speak a single word. At adult age he had a severe ID, but had a good mobility. The epilepsy (mixed seizures) persisted throughout his life despite treatment with multiple anti-epileptic drugs. Seizures were provoked by psychological stress and constipation. He had a friendly personality and loved to get attention. Ophthalmological evaluation revealed a high hypermetropia and a substantial astigmatism. The family history of the mother was positive for epilepsy without developmental

delay. At the age of 36 years conventional karyotyping and tests for Fragile X syndrome and Angelman syndrome were all normal. Upon physical examination at age 49 years his height was 175 cm (10<sup>th</sup> centile), weight was 72.5 kg (84<sup>th</sup> centile) and head circumference was 57.4 cm (<50<sup>th</sup> centile). He had minor facial dysmorphism including a high forehead, large lop ears, a prominent philtrum and a full lower lip. His teeth had been removed because of caries (fig. 1M-N). On the feet he had a clinodactyly of the second toe and a cutaneous syndactyly of the second and third toes. His motor function was clumsy and he had mild spasticity. Chromosomal analysis by 250k SNP array analysis and screening metabolic tests revealed no abnormalities. Because of his severe ID and intractable seizures mutation analysis of *SCN1A* was performed and revealed a frameshift mutation in exon 7 leading to a premature stop codon (c.3526delG (p.Glu1176fs)). Parents were not available for segregation analysis.

## Patients 7, 8 and 9: Phelan-McDermid syndrome (22qter deletion)

### Patient 7

This male patient was ascertained at the age of 48 years. He was born as the fifth child of his parents. Pregnancy, delivery and perinatal period were not remarkable. During his second year parents had noticed a delay in his psychomotor development. He learnt to walk significantly later than his siblings. His speech did not develop. At adult age his ID was severe. His behaviour was not remarkable and he had good social interactions. An abnormal breathing pattern, including intermittent irregular and rapid breathing was observed. At the age of 27 years he was diagnosed with hyperthyroidism and treatment with Strumazol and Thyrox was started, but otherwise his general health was good. His vision was reported to be mildly impaired due to mild myopia and a slightly limited visual field of unknown cause. Since the age of 45 years his general functioning had declined remarkably after an hospital admission because of severe pneumonia complicated by respiratory insufficiency. He was no longer able to walk, had feeding problems due to swallowing difficulties and became dependent on tube feeding. Contact making and social interaction diminished and he also developed seizures. Cerebral imaging by both CT scan and MRI scan showed normal brain anatomy apart from mild enlargement of the cisterna magna and central atrophy. The family history reported mild learning and behaviour problems in an older sister and severe intellectual and physical disability in a son of a brother of father.

Upon physical examination at age 48 years height was on the 50<sup>th</sup> centile. Head circumference and weight were on the 80<sup>th</sup> centile. Facial appearance was coarse with bushy eyebrows, a short philtrum, a broad mouth with very full lips and widely placed dentition and he had large ears (fig. 1O-P). He had evident syndactyly of the second and third toes, with on the right side nearly complete fusion of these toes

(fig. 2C-D). He was wheelchair dependent and showed hypertonia with spastic posture of the hands and feet. Genetic tests included chromosomal analysis by 2.7M SNP array analysis and mutation analysis of *TCF4*, because of clinical similarities with Pitt Hopkins syndrome. 2.7 M SNP array analysis identified a 1.8 Mb loss in chromosomal region 22q13.32q13.33 (47,782,571-49,543,031 Mb). Parents were not available for chromosomal analysis. Mutation analysis of *TCF4* revealed no abnormalities.

#### **Patients 8 and 9**

This brother pair was ascertained at the age of 31 and the age of 29 years respectively. Both were born after an uneventful pregnancy and delivery and had normal birth parameters. During the neonatal period the older brother (patient 8) was treated with phototherapy because of hyperbilirubinaemia. He was a very quiet baby. Since birth he had a mild spasticity of his right leg. He was treated with splints because of inverted position of his ankles. His psychomotor development was delayed. He was able to sit and walk independently respectively at the age of 12 months and 24 months. Speech development was delayed, but he learnt to speak simple sentences. Since childhood he had intermittently high levels of bilirubin and was diagnosed with Gilbert syndrome. At child age he underwent an unilateral orchidopexy because of torsio testis and his phimosis was corrected. At adult age he had a moderate to severe ID. He was diagnosed with a bipolar mood disorder. During depressive episodes he suffered from sleeping disorders and refused to eat. In general his behaviour was friendly, but regularly he showed aggressive behaviour. Social interaction was good. Upon physical examination at the age of 31 years he had a high normal height (190 cm, 75<sup>th</sup> centile), and a normal weight (50<sup>th</sup> centile) and head circumference (20<sup>th</sup> centile). Hands and feet were large-normal. Facial characteristics included a long face with prominent chin and large ears (fig. 1Q-R). He had an increased lordosis of the lumbar spine which was suggestive for muscular hypotonia of the trunk. The feet were slightly inverted.

In contrast to his brother the younger brother (patient 9) was an hyperactive baby with poor contact making. Since he was two months of age his parents noticed that he developed abnormally. The motor development was mildly delayed with sitting independently at age 13 months and walking independently at 20 months of age. Speech development was more severely delayed with the first single words at age 4 years. With age his social interaction improved and he had good contact with his parents, brother and care-takers, however contact with housemates was limited. He showed mild features of autism spectrum disorder, including obsessive behaviours. His developmental level was somewhat lower than his brother's and corresponding with a developmental level of a two to three year old child. His mood and behaviour were changeable, including periods with hyperactivity and aggressive

outbursts as well as periods with apathy. Now and then he had sleeping difficulties with awakening and wandering during the night. He was diagnosed with a bipolar mood disorder as well. At young age he underwent an operation because of cryptorchidism and phimosis. His general health condition was good. Upon physical examination he had a normal height (185 cm, 50<sup>th</sup> centile) and weight (50<sup>th</sup>-75<sup>th</sup> centile), and a small head circumference on the 2<sup>nd</sup> centile (54 cm). He had a brachycephaly. Just as his brother he had a prominent chin and large ears. In addition he had a left sided epicanthal fold, a subtle bifid point of the nose and horizontal ear lobe creases (fig.1S-T). He had a similar posture like his brother, including an increased lordosis of the lumbar spine.

Brain MRI showed in both brothers hypoplasia of the cerebellar vermis, an enlarged cisterna magna, and mild enlargement of the lateral ventricles.

Chromosomal analysis by 250k SNP array analysis identified an identical terminal loss of 2.12 Mb in chromosomal region 22q13.32q13.33 (47,35-49,47 Mb) in both brothers. Chromosomal analysis in blood lymphocytes from the parents both by SNP array analysis and FISH analysis with probes localized on chromosomal regions 22q11 and 22q13, gave normal results. To further investigate the probability of presence of the 22qter deletion in a mosaic pattern in one of the parents, we performed additional FISH experiments in buccal cells of the parents as well. Indications for a mosaic pattern in the parents were not found.

### **Evolution of phenotypes in present and previous cases**

#### **Kleefstra syndrome**

Adult patients with Kleefstra syndrome have rarely been reported before.<sup>8, 20-22</sup> Table 1 summarizes the phenotypic features of the present and 8 previously reported adult patients diagnosed with Kleefstra syndrome. Developmental outcome varied from moderate to severe ID. Most patients have little speech ability, though mostly very primitive. Five out of seven patients (in two patients presence of regression was not reported) showed some kind of regression during adolescence or adulthood, which was in three patients reported to be associated with a concurrent striking behavioural change including periods of diminished responsiveness, hypoactivity, passivity and catatonic phenomena. Five out of nine had (a history of) seizures, though in general not very severe. All adult patients had the typical facial features of Kleefstra syndrome. In the present patient the facial features were the major clue to the diagnosis in addition to the characteristic regressive behavioural pattern with onset at adult age. Microcephaly was present in four out of nine patients, three out of six had a short adult height and three out of seven patients presented with obesity. In conclusion, most important diagnostic clues at adult age seem to be the typical facial features and behaviour pattern which might be associated with regression of general functioning.

**Table 1** Clinical features in present and previously reported patients with (genetically confirmed) Kleefstra syndrome.

	Present patient	References previous reports				Total
		8	20-21	22, 41	22	
Number of adults	1	1	6	4 (excl. present patient 1)	1 (other 2 adults are included in <sup>41</sup> )	13
Genetic defect	<i>EHMT1</i> mutation: c.3087 +1 G >T	Microdeletion	6/6 microdeletion	3/4 microdeletion 1/4 <i>EHMT</i> mutation	Microdeletion	11/13 microdeletion 2/13 <i>EHMT1</i> mutation
Age, years	41	36	20–59	18-32	19	18–59
Gender	f	F	4 m, 2 f	3 f, 1 m	f	5 m, 8 f
Developmental outcome	moderate ID, some speech	severe ID	6/6 variable ID	Moderate--severe ID	Moderate ID	13/13 moderate--severe ID
Motor symptoms	Focal myclonic twitches shoulder, fixed flexure arms and hands	NR	1/2 progressively immobile 1/2 frequent myoclonic twitches, slight hypertonia fixed flexure arms and hands in 2/2	1/4 rigid flexure of the arms and hands	Slow, perseverative and clumsy motor functioning at adult age	In two patients myoclonic twitches In 1 patient immobility In 4 fixed flexure extremities
Regression (of psychomotor function)	+	NR	3/5	1/4	+	6/11
Behavior/psychiatric	Impulsivity and aggressive outbursts. Around age 38 remarkable behavior change: periods of apathy, decreased emotional responsiveness, staring, low motor activity, stupor, and sleep disturbance	+, not further specified	2/5 aggression 2/5 sleep disturbance 3/5 passive periods 2/5 mood disorder 2/2 stereotypic orofacial movements 3/5 increase of behavior problems/ temporary regression during teenage years 2/2 during midlife occurrence of bizarre and distinct posture, hypoactivity, minimal emotional response, lack of initiative, and staring	1/4 sleep disturbance 1/4 stereotypies 2/4 mood disorder 1/4 hyperactivity 1/4 increase of behavior problems during teenage years with progressive inactivity and catatonic features	Sleep disturbance Increase of behavior problems in adolescence with later on general inactivity and irritability Stereotypies	Variable behavior problems in all; temporary (behavioral) regression/increase of behavioral problems during adolescence; onset of typical behavior pattern at middle age including periods with passivity, decreased responsiveness and hypoactivity in a subset.
Seizures	–	+	4/6	2/4	-	7/13
Recognizable facial features	+	+	+	+	+	present in all
Medical problems, age of onset (when documented)	Hypertrophy of the left ventricle and paroxysmal atrial fibrillation, diagnosed around age 40 years	NR	1/6 scoliosis 1/6 severe GOR with weight loss 1/6 pyloric stenosis 1/6 death with unknown cause at age 21	2/4 frequent respiratory infections/aspiration, 1/4 mild pulmonic regurgitation as an adult 1/4 scoliosis	Ventricular septum defect	3/12 cardiac 2/12 scoliosis 2/12 gastro-intestinal 1/12 early death 2/12 recurrent respiratory problems

Table 1 Continued.

	Present patient	References previous reports				Total
		8	20-21	22, 41	22	
Adult growth parameters						
head circumference	normal	Normal	3/6 microcephaly	1/3 microcephaly	microcephaly	5/13 microcephaly
height	normal	NR	2/4 short stature	2/4 short stature	Short stature	5/10 short stature
weight	obesity	Obesity	1/5 obesity	1/4 obesity	obesity	5/12 obesity
Other	brain MRI: poorly defined abnormal white matter signal intensities, mainly localized deep in the parietal lobe, bilaterally abnormal signal intensity in the globus pallidus	Brachydactyly	1/6 hearing loss 2/6 cryptorchidism 1/6 VUR 3/5 minor anomalies on brain MRI: 1/5 small pons, 1/5 prominent Virchow-Robin spaces, 1/5 asymmetry of anterior horns	1/4 Hearing loss 1/4 brachydactyly 1/4 hyperopia 1/4 cryptorchidism 1/3 white matter lesions, corpus callosum hypoplasia	-	5/10 variable anomalies on brain MRI

NR = not reported; GOR = gastro-oesophageal reflux; VUR = vesicoureteral reflux

Pitt Hopkins syndrome

The present patient is the oldest reported patient with Pitt Hopkins syndrome (PHS). As far as we know, 7 adult patients with genetically confirmed PHS have been reported in previous studies (Table 2). All adults with PHS had a severely impaired developmental outcome. A minority suffered from seizures that presented at childhood age. Late-onset seizures were not reported, suggesting that if seizures develop, they present at young age. Medical problems were relatively mild, including mainly constipation, scoliosis and aspecific ocular problems, though one patient died prematurely from pneumonia. Motor functioning was mildly impaired in some cases. Reported behavioural problems are diverse, but most patients have a happy disposition. Among this small group of adults with PHS there are no indications for significant regression at adult age. The typical facial characteristics persist during adult age. In the present patient his facial gestalt was the clue to the diagnosis. In general, the combination of the typical facial characteristics, severe ID, the breathing pattern with periods of hyperventilation followed by apnoea, and the clubbing of finger and toe nails might suggest the diagnosis PHS in adults.

MECP mutations in males

MECP2 mutations are involved in Rett syndrome. Initially it was thought that Rett syndrome occurs exclusively in females due to lethality of hemizygous mutations in males. Though, after the identification of the MECP2 gene in 1999, several MECP2 mutations have been detected in males as well. The phenotypic variability in males with MECP2 mutations is wide and roughly three groups of phenotypes can be observed: 1) classical/atypical Rett syndrome occurring in males with a classical Rett syndrome mutation in mosaic pattern or in males with an XXY karyotype 2) severe congenital encephalopathy, mostly associated with a mutation that causes Rett syndrome in females and 3) a broad group with wide phenotypic spectrum including variable levels of ID and variable occurrence of other neurological and/or psychiatric disorders.<sup>23-25</sup> The phenotypes caused by MECP2 duplications are not considered here. Mutations that cause the classical Rett syndrome phenotype in females are considered to be either prenatally lethal in males or leading to severe congenital encephalopathy and early death.<sup>26</sup> The present patient can be assigned to the third group. Several males from X-linked ID families and single male patients with a phenotype fitting in the third group have been reported before.<sup>27-30</sup> Frameshift/truncating mutations, as observed in the present patient infrequently have been reported in males before (2 previous reports).<sup>23 31</sup> Adult phenotypes of present and

**Table 2** Clinical features in present and previously reported patients with (genetically confirmed) Pitt Hopkins syndrome.

	Present patient	References previous reports			Total
		9	42	43	
Number of adults	1	2	3	1	7
Genetic defect	<i>TCF4</i> mutation: c.1739G>A	<i>TCF4</i> mutations: c.1153 C>T and IVS9–1G>C	<i>TCF4</i> mutations: c.1699A>T, c.469C>T, c.1073G>T	64-kb microdeletion 18q21.2	6 <i>TCF4</i> mutations, 1 small microdeletion
Age, years	40	29	18--20	19	18--40
Developmental outcome	severe ID	severe ID	severe ID	severe ID	severe ID
Motor symptoms	mild hypertonia	1/2 ataxia 1/2 wide based walking pattern	1/3 ataxia	walks with assistance	2/7 ataxia, 1/7 hypertonia
Regression (of psychomotor function)	–	NR	NR	NR	never reported
Behavior/psychiatric	happy disposition, pica	1/2 very anxious, self mutilation	2/3 happy disposition 1/3 violent outburst, stereotypies	happy disposition, bangs on objects, sleeping problems	mostly happy disposition, but diverse behavior problems do also occur
Seizures	–	–	1/3, since 9 months of age	–	2/7, onset during childhood
Recognizable facial features	+	+	2/3 + 1/3 +/-	+	7/7
Medical problems, age of onset (when documented)	myopia, scoliosis, osteoporosis and atopic constitution, onset in childhood	1/2 severe constipation, and strabismus in, onset NR 1/2 lymphoma and dislocated hip, onset NR	2/3 constipation, scoliosis 1/3 myopia, strabismus	strabismus, myopia	constipation, scoliosis, and aspecific ocular problems in several patients
Adult growth parameters					
head circumference	P0.6	2/2 <P3	2/3 <P3, 1/3 normal	P50	5/7 microcephaly
height	>P2	2/2 <P3	NR	P10–25	3/4 short stature
weight	P16	2/2 <P3	NR	P60	2/4 underweight
Abnormal breathing pattern <sup>a</sup>	–	2/2	2/3	+, breath holding	5/7
Other	finger and toe nail clubbing	finger and toe nail clubbing in 1/2	NR	NR	2/3 nail clubbing

NR = not reported.

P = Percentile.

<sup>a</sup>Comprising periods with hyperventilation followed by apnoea.



23 previous cases are summarized in Table 3. Age ranged from 21 to 56 years. All have an impaired developmental outcome, though varying from mild to profound ID. From these data a significant phenotype-genotype correlation with respect to the degree of ID cannot be observed. In two of the families reported, identical mutations lead to a developmental level varying from mild to severe disability.<sup>29, 32-33</sup> Almost all patients showed evident motor/neurological symptoms, as tremors (46%), pyramidal signs (58%) and in two patients ataxic signs were observed. Interestingly, in one family tremors were reported in the carrier females as well.<sup>29, 33</sup> Almost 30% of the males showed regression in functioning at adult age. One third of the patients had seizures, with the exception of one, onset was reported at childhood age. A majority (64%) had microcephaly and short stature (67%). One out of six developed kyphosis/scoliosis with age. Many were reported to have a friendly and cooperative behaviour, though a few patients showed aggressive outburst. Mood/depression and anxiety disorders were frequently observed. A subset of the patients had a passive, inactive personality and some were shy/introverted in social interaction. In conclusion, clue features that might indicate presence of a *MECP2* mutation in adult males are: variable ID in combination with neurological symptoms, including pyramidal signs and tremors, mood and anxiety disorders and possibly regression at adult age. Facial features are not remarkable and do not contribute to the diagnosis.

#### ***Rett syndrome variant with infantile spasms (CDKL5 mutations)***

The present patient is the oldest patient with a mutation in *CDKL5* reported. Table 4 summarizes the clinical features of the present and 9 other adult female patients.<sup>7, 34-38</sup> The developmental outcome is very poor. Remarkably, in one patient (twin sister of severely affected patient) the developmental level was reported to be only mildly impaired.<sup>38</sup> Six out of ten patients were not able to speak. All patients showed motor symptoms, including mostly pyramidal signs, but also cerebellar symptoms were reported. Secondary locomotor tract deformities including scoliosis and/or contractures were present in six out of eight patients. Five out of nine patients had signs of autonomic dysregulation (intestinal symptoms, breathing anomalies and vasomotor disturbance). Regression was reported in 4 out of 10 patients. Seizure outcome was very poor. Nine patients suffered from persistent intractable seizures into adulthood. Behaviour characteristics that were frequently present included (hand)stereotypies and poor social interaction. Sleeping problems were documented in four patients. Small hands and/or feet were also more often reported (in 4/7).

In conclusion, the consistent clinical features at adult age included a very poor developmental and seizure outcome in combination with other neurological signs including pyramidal tract and cerebellar signs and autonomic signs. Major health

problems were secondary to neurological problems. Behaviour symptoms included Rett-like behaviours, such as (hand)stereotypies.

#### ***Dravet syndrome***

In contrast to the other reported syndromes in the present study, follow-up of Dravet syndrome patients into adulthood has more often been documented before.<sup>39-40</sup> The phenotype of 37 adult patients, including the present two patients, is summarized in table 5. Age ranged from 18 to 49 years. The majority had a poor developmental outcome with severe ID in more than 80%. Seventy-five percent had motor symptoms (including pyramidal, cerebellar and extrapyramidal symptoms), varying from clumsiness to bedridden. Regression was uncommon. In 86% intractable seizures with a history of early onset in the first year of life, persisted into adulthood. Behaviour characteristics were not systematically reported, though the present two patients both had a friendly and cooperative personality. Medical problems were not systematically reported.

Poor developmental and seizure outcome in combination with motor symptoms, but lack of other specific features might be indicative of Dravet syndrome in adult male and female patients.

#### ***Phelan-McDermid syndrome***

Clinical features of the present patients and 15 previously reported adult patients are summarized in Table VI. Age ranged from 19 to 48 years. The present patient 7 is the oldest patient reported. Developmental outcome varied from mild to severe ID. Most patients did have some speech ability. We observed a tendency to deterioration in motor functioning above the age of 40 years, including presence of pyramidal signs. The present patient 7 showed a severe decline in motor functioning, as well as in cognitive and health performance.

About half of the patients did at least have one seizure during lifetime, but severe seizure disorders were not reported. The majority of the adult patients had variable behaviour problems, including autistic behavioural features, though in some patients these seemed to improve with age. Other features that were more frequently reported included a long face and large ears, large and/or slender hands/feet and hypotonia with/without lumbar lordosis.

In four patients, including the present patients 8 and 9, results of cerebral imaging by MRI were reported. Interestingly, in three of these patients similar anomalies, comprising cerebellar vermis hypoplasia, enlargement of the cisterna magna and dilatation of ventricles were observed.

**Table 3** Clinical features of present and previously reported male patients with 'mild' *MECP2* mutations that do not cause classical Rett syndrome in female patients or severe neonatal encephalopathy/prenatal death in males.

	Present patient	References previous reports						Total
		29, 32-33 (MRX16)	30, 33 (T44)	29, 33 (T36)	28 (X307, MR48, MR50, X308)	44-45	27, 46	
Number of adults	1	7	3	4	4	4	1	24
Mutation/domain	frameshift: c.1233_1243del (p.Ser411fs)/C-terminus	missense: c.410A>G (p.Glu137Gly)/MBD	in frame deletion c.1161_1400del/C-terminus	missense: c.499C>T (p.Arg167Trp)/Co-repressor interacting region	missense: c.493 C>T (p. Ala140Val)/MBD	missense: c. 493 C>T (p.Ala140Val)/MBD	nonsense: c.1216 C>T (p.Glu406X)/C-terminus	19/24 missense 1/24 nonsense, 1/24 frameshift, 3/24 in frame deletion 5/24 in MBD 5/24 in C-terminus 4/24 in co-repressor interacting region
Age, years	27	21–56	24–55	25–50	27–40	25–41	39	21–56
Developmental outcome	severe ID	6/7 mild/moderate ID 1/7 profound ID	1/3 mild ID 1/3 moderate ID 1/3 severe ID	4/4 mild ID	4/4 severe ID	4/4 moderate ID	severe ID	24/24 variable ID
Motor symptoms	mild spasticity, hypertonia, brisk reflexes, generalized muscular atrophy; frequent falling; resting tremor.	5/6 hyperreflexia (1 NR)	1/3 hypertonia	3/4 resting tremor 3/4 hyperreflexia 1/4 ataxia	4/4 resting tremor	3/4 resting tremor 3/4 hyperreflexia 2/4 hypertonia	spasticity, hyperreflexia, choreoathetotic movements, ataxic gait	11/24 tremor 14/24 pyramidal signs 2/24 ataxia
Regression (of psychomotor function)	+, global decline since early twenties	4/6 language egression	–	1/4 deterioration of articulation	–	–	+, around age 30 motor decline/progressive spasticity	7/24
Behavior/psychiatric	passivity, lack of initiative, cooperative, calm, shy and friendly; depression, sleep disturbance	4/7 introvert/timid/ shy 3/7 anxiety 2/7 passivity/slowness	1/3 irritability, depression, emotional disturbance 1/3 poor contact and violence 1/3 shy, quiet, anxious, and attention problems	3/4 pleasant 1/4 aggression 1/4 anxiety	slow, friendly	3/4 depression 2/4 aggression	NR	6/23 shy/timid 10/23 mood/anxiety disorder 4/23 aggression 4/23 passivity/hypoactivity
Seizures	+, grand mal, onset age 12	1/7, single seizure	1/3	2/4	NR	1/4	+, grand mal onset age 27	7/20
Recognizable facial features	–	–	–	NR	NR	–	–	Not present

Table 3 Continued.

	Present patient	References previous reports						Total
		29, 32-33 (MRX16)	30, 33 (T44)	29, 33 (T36)	28 (X307, MR48, MR50, X308)	44-45	27, 46	
Medical problems, age of onset (when documented)	–	1/7 severe myopia 3/7 kyphosis/scoliosis	NR	NR	NR	1/4 congestive heart failure, onset age 39 1/4 kyphoscoliosis	bilateral cataract age 30, episodes of unexplained diarrhea; death at age 39 from pneumonia	variable, 4/24 kyphosis/scoliosis
Adult growth parameters								
head circumference	P25–P50	3/7 <P3	1/3 normal, 2/3 NR	2/4 <P3	4/4 normal	4/4 normal	Normal	5/22 <P3
height	P16	6/7 <P3	2/3 normal, 1/3 NR	4/4 <P3	NR	2/4 short stature	NR	12/18 short stature
weight	P2	>P98 in 2/5	NR	3/4 obesity	NR	1/4 obesity	NR	6/14 obesity
Other	–	–	–	–	–	macro-orchidism, pyramidal signs	siallorhoe	no consistent other features
Family history	learning disabilities in mother and sister, mother has severe psychiatric problems	patients are from the same family, carrier females all normal ] intelligent	4 affected males in one family, no information about carrier females	progressive tremor in 2 carrier females, normal intelligence in carrier females	mother and carrier sister have mild ID	mother low intelligence	borderline intelligence in 1 of 2 carrier females; severe ID in nephew	all familial cases; in 4/6 families carrier females have mild ID/learning difficulties (NR in 1 family)

MBD = methyl binding domain; P = Percentile; MRX/TX/MR numbers refer to the family identification numbers in the original papers

**Table 4** Clinical features in present and previously reported patients with *CDKL5* mutations.

	Present patient	References previous reports						Total
		38	38	7	34	35-36	37	
Number of adults	1	2	1	2	1	2	1	10
Mutation	c.464-1G>A	c.183delT	IVS13-1G>A	525A>T (R175S)	c.del2362_2366	C1892.1893dupTA/ c.229delGAAG	Del exon 1a-3	
Age, years	47	19 (twins)	28	41 (twins)	18	19, 26	20	18-47
Sex	f	f	F	f	f	f	f	10 f
Developmental outcome	severe ID, no speech	Severe ID, no speech in twin 1, mild ID in twin 2	severe ID, no speech	severe ID in both, no speech	severe ID, speech in phrases	severe ID, first patient able to speak 3 word sentences, second patient no speech	severe ID, no speech	9/10 severe ID 1/10 mild ID (speech is rare)
Motor signs	spastic tetraplegia, unable to walk without support, ataxia	wheel chair bound, generalized spasticity, hyperreflexia (twin 1)	unsteady gait	one is wheel chair bound, twin sister is able to walk since age 8 with support. In both mild ataxia	motor dyspraxia, limited hand skills, hypotonia, but walks and swims	unable to walk, limited hand skills	wheelchair bound, hyporeflexia	10/10, comprising pyramidal and sometimes cerebellar components
Regression (of psychomotor function)	+, decline motor functioning	twin 1: + normal development until 10 mo, then loss of skills	+, between age 2-5 years loss of skills	not significant	-	-	+, between ages 4-7 years	4/10
Behavior/psychiatric	self mutilation, stereotypic movements (hand biting), hyperactivity, no sense of fear	twin 1: poor eye contact, hand stereotypes	hand stereotypes, dystonia, sleep disturbances	hand stereotypes, mood swings, little eye contact and interaction with environment in both	hand stereotypes, autism, bruxism, mood lability, sleep disturbance	hand stereotypes, autistic features, sleep disturbances (both). Bruxism (2)	NR	8/9 hand stereotypes 6/9 poor social interaction/autism 4/9 sleep disturbance
Seizures								
onset	6 months	1/2: 9 weeks	6 weeks	10 weeks	2 months	6-12 weeks	first 2 weeks of life,	
type and severity, therapy resistance	atonic, intractable; persistent seizures despite of multiple medication	1/2 infantile spasms, later mixed <sup>a</sup> seizure disorder 1/2: no seizures	severe infantile spasms, later mixed intractable seizures	infantile spasms until 6 months in both, later absences (in one of the twins)	West syndrome, subsequent severe mixed seizure disorder	no infantile spasms 1/2 refractory seizures 1/2 seizure arrest at 2 weeks	+, mixed, intractable and therapy resistant	9/10 9/9 early onset 8/9 persistent severe seizure disorder into adulthood
Facial features	coarseness	NR	no dysmorphism	NR	NR	NR	coarseness, bilateral exotropia	2/3 coarseness (possibly due to anti-epileptic medication)
Medical problems, age of onset	Pes equinovarus (related to spasticity), gastrostomy tube	thoracolumbal scoliosis	scoliosis	progressive dorso-lumbal scoliosis in and flexion contractures in both	-	NR	gastrostomy tube	6/8 locomotor tract problems secondary to spasticity 2 patients with gastrostomy because of feeding difficulties

Table 4 Continued.								
Adult growth parameters								
head circumference	<P16	1/2 normal	NR	2/2 P5--P15	normal	2/2 microcephaly	normal	2/9 microcephaly
height	<P16	1/2 <P3 1/2 P3–P10	NR	NR	normal	NR	normal	1/8 short stature
weight	P2	1/2 <P3 1/2 P10–P25	NR	NR	NR	NR	normal	2/6 cachexia
Autonomic features	constipation	1/2: breathing abnormalities, peripheral vasomotor disturbance; severe constipation	+	episodes of hyperventilation, abdominal distention in both	–	1/2	NR	5/9
Other	small hands, corpus callosum agenesis	small hand and feet; male brother also affected, died at age 16 of respiratory failure	small feet, hirsutism	NR	NR	NR	acne, hirsutism	4/7 small hands and/or feet

P = Percentile; \* Mixed: tonic-clonic, myoclonic, absences.



**Table 5** Clinical features of present and previously reported male patients with genetically confirmed Dravet syndrome.

	Present patient 5	Present patient 6	References previous report		Total
			39	40	
Number of patients	1	1	10	25	37
Mutation	c.5304T>G (p.Ser1768Arg)	c.3526delG (p.Glu1176fs)	NR	14/25 missense 5/25 nonsense 6/25 frameshift	Missense > frameshift > nonsense
Age, years	34	49	mean 26 (range 18--47)	18--43	18--49
Gender	M	m	NR	9 males, 16 females	11 males, 16 females, 10 NR
Developmental outcome	severe ID, no speech	severe ID, very little speech	1/10 normal 4/10 moderate ID 5/10 severe ID	24/25 severe ID 6/25 no speech 8/25 very limited speech 6/25 single words 4/25 simple speech 1/25 mild ID	31/37 severe ID 4/37 moderate ID 1/37 mild ID 1/37 no ID
Motor symptoms	mild right-sided hemiplegia, after epileptic state, able to walk	clumsiness, spasticity	5/10 pyramidal 3/10 cerebellar 4/10 extrapyramidal 2/10 no motor signs	2/25 bedridden 11/25 clumsy 7/25 ataxia 5/25 no motor signs	30/37 motor signs, comprising pyramidal, cerebellar and extra-pyramidal signs
Regression	+, onset at age 2 years	–	uncommon	5/25	6/27
Behavior/psychiatric	very friendly, little initiative, obsessive traits, self mutilation, very high threshold of pain	friendly personality, loves to get attention	NR	NR	2/2 cooperative and friendly personality
Seizures					
age at onset	5 months	6 months	3--8 months	<first year of life	37/37 onset within first year of life
seizure types	absences, tonic-clonic	mixed	at onset: 1/10 myclonic ,4/10 generalized tonic-clonic, 5/10 febrile; later: mixed seizures in 8/10 (tonic-clonic, absences, myoclonic, partial)	generalized at adult age, only in 6 mixed seizures	variable types of seizures
severity	once epileptic state, intractable seizures despite of multiple anti-epileptic drugs	intractable seizures despite of multiple anti-epileptic drugs	intractable seizures in 10/10	intractable in 20/25. In 5 seizures suppressed for 1--3 years	32/37 intractable seizures persisting throughout adulthood
Recognizable facial features	–	–	NR	NR	not present
Medical problems	upper and lower airway infections	high hypermetropia and a substantial astigmatism	NR	1 patient described as case report: pneumonia	2/3 airway infection (not systematically reported)
Growth parameters	Normal	normal	NR	NR	12/12 normal growth parameters

NR = not reported.

**Table 6** Clinical features of present and previously reported patients with Phelan-Mc Dermid syndrome.

	Present patient 7	Present patients 8, 9	References previous reports								Total
			47	48	49	50	51	52	53	54	
Number of adults	1	2	1	1	1	1 (from brother pair)	2	1	7	1	18
Genetic defect	1.8-Mb deletion	2.12-Mb deletion	130-kb terminal deletion	submicroscopic deletion	100-kb terminal 22q13.3 deletion	3.5-Mb deletion	5.65-Mb and 5.79-Mb deletions	intragenic SHANK3 deletion due to translocation Xq21.33 and 22q13.33	6 with r(22), 1 with t(22)	46,XX, der(22)t(14;22)q32.31;q13.33	9/18 22q13.3 1 intragenic SHANK3 deletion 6/18 ring chromosome 22 2/18 balanced translocation
Age, years	48	31, 29	22	26	33	24	19, 31	20	20–36	46	19–48
Gender	m	m	f	f	F	m	m, f	f	3 m, 4 f	f	8 m, 10 f
Developmental outcome	severe ID, no speech	moderate-severe ID	severe ID, no speech	learning difficulties	mild ID	severe ID, limited speech	significant speech delay	moderate ID, simple speech, difficult to understand	7/7, in 2/7 severe (all r(22)).	+	18/18, variable, most have some speech
Motor signs	hypertonia, progressive spasticity and wheelchair bound after age 45; swallowing difficulties after age 45	1/2 mild spasticity right leg 2/2 truncal hypotonia	fully mobile	NR	ataxic gait	hypotonia	NR	oral: poor articulation	5/7 hypotonia	hyperreflexia and clonus, ataxia, decreased ambulation	tendency to deterioration in motor functioning above age 40, with onset of pyramidal signs
Regression	+, general decline in functioning	no	NR	NR	+, at adult age, decline in speech, motor function, daily living functions, incontinence	NR	NR	NR	NR	+? limited early history	2–3/18 (at age >35 years)
Behavior/psychiatric	not remarkable	bipolar mood disorder, sleep disturbance and high threshold of pain in both. Mild features of autism spectrum disorder, improving with age (pat. 9)	NR	NR	autistic features: lack in contact, stereotypic movements	quiet and loving, but stubborn; peculiar catatonic like episodes, high threshold of pain	+, not further specified	hand stereotypies, sometimes irritated and aggressive; more social and less ritualistic than in childhood	7/7 very severe	NR	14/15 variable, tendency to improvement of social interactions with age?

Table 6 Continued.

	Present patient 7	Present patients 8, 9	References previous reports								Total
			47	48	49	50	51	52	53	54	
Seizures	+, onset at age 45 y	no	NR	NR	+	+, febrile, incidental	1: no 2: yes	NR	2/7	+, focal and petit mal	7/15
Facial dysmorphic features	coarse, bushy eyebrows, short philtrum, broad mouth, full lips, widely placed dentition, large ears	1/2 long face 2/2 prominent chin, large ears	microgna-thia, simple ears, prominent nose, gap between incisors	wide broad forehead, bulbous nose tip, prominent columella, high palate	long face, high nasal bridge, broad nose	asymmetry of the face, midface hypoplasia, square forehead, prominent supraorbital ridges, bulbous tip of the nose, short philtrum, large ears, prominent lower lip, high arched palate, gap between incisors	NR	periorbital puffiness, broad nasal bridge, slightly low-set and outwardly rotated ears, high and narrow palate, narrow bitemporal diameter, micro-retrognathia, low hairline neck	5/7 dysplastic ears	narrowed bitemporal diameter, epicanthal folds, periorbital fullness, flat midface	long face, large ears; midface hypoplasia, periorbital fullness are more often reported
Medical problems, age of onset (when documented)	hyper-thyroidism since age 27 years	Gilbert syndrome (pat. 8) since childhood	–	mitral valve prolaps, diagnosed at age 18 years	–	right convex scoliosis, diagnosed at age 22 years; constipation, since first years of life	recurrent upper respiratory tract infections in 1/2	–	NR	NR	variable
Growth parameters <sup>a</sup>											
height	P50	P75 (pat. 8); P50 (pat. 9)	NR	P75-90	slender build	P25	1: accelerated, 2: normal	P75	6/7 normal 1/6 short stature	NR	normal-increased in majority
weight	P80	P50 (pat. 8); P50–75 (pat. 9)	NR	NR	NR	>P97	NR	P90	NR	NR	normal-increased in majority
head circumference	P80	P20 (pat. 8); P2 (pat. 9)	NR	>P97	NR	>>P98	NR	P10	NR	NR	normal-increased in majority

Table 6 Continued.

	Present patient 7	Present patients 8, 9	References previous reports								Total
			47	48	49	50	51	52	53	54	
Other	intermittent irregular and rapid breathing. 2/3 syndactyly of the toes	1/2 endorotation ankles, normal-large hands and feet 2/2 genital anomalies 2/2 on brain MRI hypoplasia of the cerebellar vermis, enlarged cisterna magna, mild dilatation lateral ventricles	2/3 long, thin fingers, 2/3 syndactyly of the toes, club feet deformity	long thin fingers, nasal speech, unusual hair texture, recurrent miscarriages.	long fingers and toes, normal brain MRI	large hands and feet, persistence of fetal finger pads. 2/3 syndactyly of the toes brain MRI: cortical atrophy, pachygyria, cerebellar vermis hypoplasia, enlarged cisterna and ventricles	NR	prominent lumbar lordosis; slender hands with mild webbing between fingers, webbing between 2/3 toes	NR	NR	lumbar lordosis, probably related to truncal hypotonia 2/3 syndactyly toes, large and/or slender hands. 3/4 similar anomalies on brain MRI

NR = not reported; P = percentile.

**Table 7** Distinguishing features in adult patients with different Angelman- and Rett-like syndromes.

	Kleefstra syndrome	Pitt Hopkins syndrome	MECP2 mutations in males	CDKL5 mutations	Dravet syndrome	Phelan-McDermid syndrome
Facial appearance	recognizable	recognizable	non-recognizable	non-recognizable	non-recognizable	subtle, including long face, large ears
Epilepsy	+/-, mild	+/-, mild	+/-, mild	+, intractable	+, intractable	+/-, mild
Motor symptoms	+/-, catatonic phenomena	+/- mild	+, pyramidal, tremors, cerebellar	+, pyramidal, cerebellar	+, pyramidal, cerebellar	–
Degree of ID	moderate–severe	Severe	mild–profound	mostly severe	severe	mild–severe
Regression	+ (6/11), at adult age	–	+ (1/3) at adult age	+ (4/10)	–	–
Typical behavior / psychiatric features	adult age onset periods with passivity, decreased responsiveness and hypoactivity; sleep disturbance	mostly happy disposition, but diverse behavior problems including pica and self mutilation	mood/depression and anxiety disorders; shyness and passivity	(hand)stereotypies and poor social interaction	friendly and cooperative in general	autistic features, mood disorders
Signs of autonomic dysfunction	–	abnormal breathing pattern, constipation	–	breathing anomalies, cold and purple feet/vasomotor disturbance, severe constipation	–	+/- abnormal breathing pattern in present patient 7
Growth parameters	Microcephaly, short stature and obesity in about 40-50%	microcephaly and short stature in majority	microcephaly and short stature in majority	normal–decreased	normal	normal–increased
Medical problems and/or other distinctive features	no consistently occurring medical problems	typical clubbing of finger and toe nails	spinal column deformity	spinal column deformity, scoliosis; small hands and/or feet; feeding problems/ gastrostomy	airway infections more often reported	long and/or slender fingers/toes, 2/3 syndactyly toes
Life expectancy (oldest reported patient, years)	59	40	56	47	49	48

Discussion

The adult phenotype of most of the presented syndromes has not been systematically reviewed and described in previous reports, except for *SCN1A* mutations, involved in Dravet syndrome.<sup>39-40</sup>

We described the phenotype of 9 adults with 6 different Angelman- and Rett-like syndromes and reviewed previously reported adult cases in literature. As in childhood patients, adult patients with the reported Angelman- and Rett-like syndromes showed an overlapping phenotypic spectrum as well. Despite the relatively low numbers of patients in each group that have been reported thus far, a tendency to specific phenotypic differences at adult age, with respect to evolution

of the phenotype and outcome, could be observed, though conclusions are hampered by the small numbers (Table 7). Patients with Kleefstra syndrome, males with *MECP2* mutations and females with *CDKL5* mutations, showed a further decline in functioning/regression at adult age, whereas patients with Pitt Hopkins syndrome, Phelan-McDermid syndrome and Dravet syndrome seemed to have a relatively stable performance. The prognosis with regard to the outcome of epilepsy showed also significant differences, with the poorest outcome in Dravet syndrome patients and patients with *CDKL5* mutations. Motor symptoms were clearly present in some syndromes, including male Rett syndrome, Kleefstra syndrome (catatonic phenomena) and in patients with *CDKL5* mutations, but absent or minor in other syndromes. Distinctive behaviour patterns could be observed as well, for example



adult onset of periods with passivity, decreased responsiveness and hypoactivity in Kleefstra syndrome and Rett syndrome like behaviours in patients with *CDKL5* mutations. Major medical problems, except for severe epilepsy, were infrequently reported in adult patients with the presented Angelman- and Rett-like syndromes. Observed medical problems were mostly secondary to motor complications, for example secondary scoliosis, contractures, feeding difficulties and airway infections due to spasticity, which were predominantly observed in patients with *CDKL5* mutations, *MECP2* mutations and *SCN1A* mutations.

Patients with Kleefstra syndrome and Pitt Hopkins syndrome retain the recognizable facial features at adult age, and thus, in these patients their facial appearance is an important clue to the diagnosis.

In conclusion, the combination of different key clinical features at adult age might be helpful to distinguish specific Angelman- and Rett-like syndromes from each other. These key features comprise the degree of ID, seizure characteristics, motor symptoms, occurrence of regression and characteristic facial appearance (Pitt Hopkins syndrome and Kleefstra syndrome). Knowledge about and insight in syndrome specific adult clinical characteristics facilitates adequate management and follow-up of patients with Angelman- and Rett-like syndromes, and enables careful counseling of family members regarding prognosis, natural course of the disease and life expectancy.

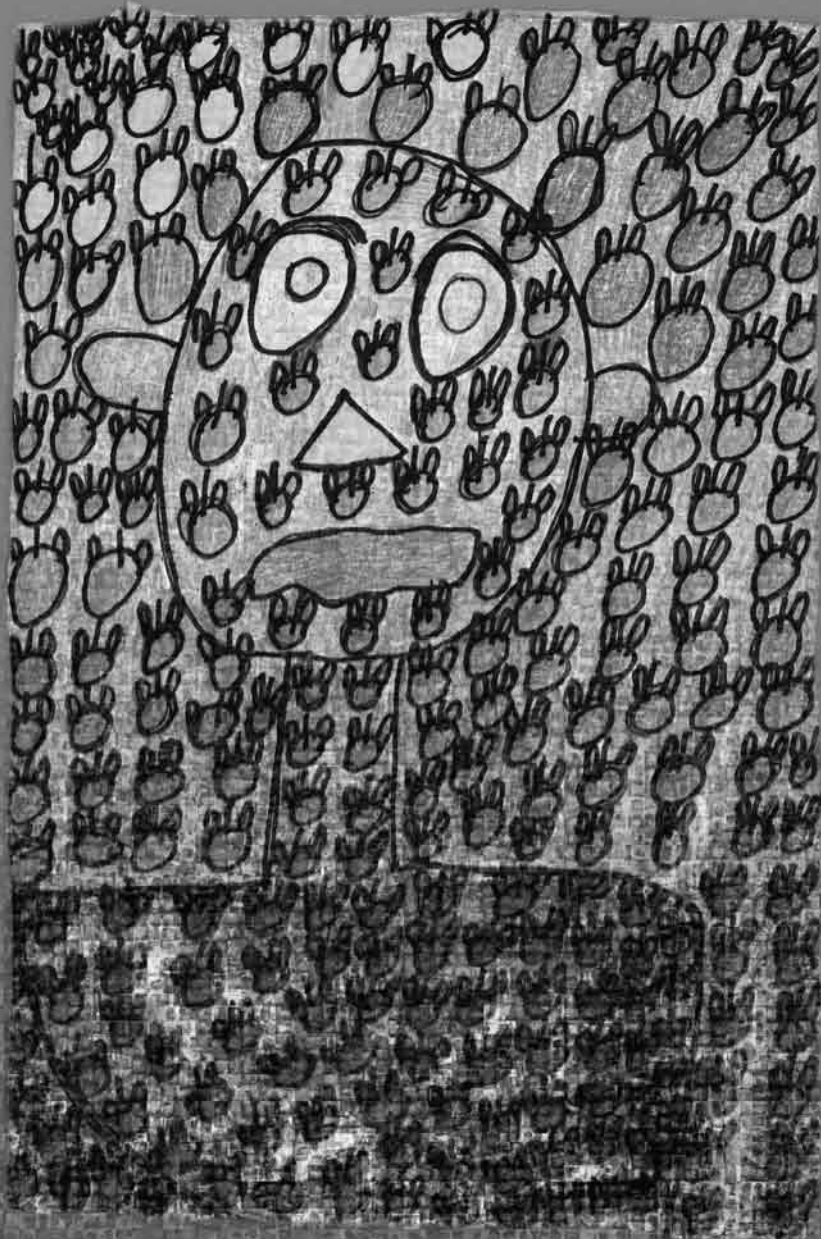
### Acknowledgements

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# 5

## Exome sequencing in sporadic and familial ID

5.1 Mutations in *DYNC1H1* cause severe intellectual disability with neuronal migration defects and peripheral neuropathy  
*J Med Genet* 2012;49:179-183

5.2 Diagnostic exome sequencing in patients with severe intellectual disability  
*N Engl J Med*, in Press

5.3 Involvement of the kinesin family members *KIF4A* and *KIF5C* in intellectual disability and synaptic function  
*Submitted*





# 5.1

## **Mutations in *DYNC1H1* cause severe intellectual disability with neuronal migration defects**

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## Abstract

*DYNC1H1* encodes the heavy chain protein of the cytoplasmic dynein 1 motor protein complex that plays a key role in retrograde axonal transport in neurons. Furthermore, it interacts with the *LIS1* gene of which haploinsufficiency causes a severe neuronal migration disorder in humans, known as classical lissencephaly or Miller-Dieker syndrome. We identified *de novo* missense mutations in *DYNC1H1* (p.Glu1518Lys and p.His3822Pro) in two patients with severe intellectual disability and variable neuronal migration defects. An autosomal dominant mutation in *DYNC1H1* was previously identified in a family with the axonal (type 2) form of Charcot- Marie-Tooth (CMT2) disease. Mutations in *Dync1h1* in mice also cause impaired neuronal migration in addition to neuropathy. Our data suggest that mutations in *DYNC1H1* can lead to a broad phenotypic spectrum and confirm the importance of *DYNC1H1* in both central and peripheral neuronal functions.

Neuronal migration disorders are an heterogeneous group of neurodevelopmental disorders that represent an important cause of intellectual disability (ID) and epilepsy in humans. Lissencephaly is due to failure of migration of neuronal precursors from the paraventricular zone to the cerebral cortex during early brain development.<sup>1-3</sup> Lissencephaly is classified in several subtypes, one of which is classical lissencephaly.<sup>1</sup> Classical lissencephaly can be present as an endophenotype in contiguous gene syndromes, such as Miller Dieker syndrome (deletion chromosome 17p13) (MDLS (MIM 247200)), but can also be present in an isolated form. The vast majority of classical lissencephaly is caused by haploinsufficiency of the *Lissencephaly 1(LIS1)/Platelet-activating factor acetylhydrolase, isoform 1B, alpha subunit (PAFAH1B)* [MIM 601545] gene or mutations in the gene *doublecortin (DCX)* (MIM 300121)) on the X-chromosome.<sup>1, 4</sup> Patients with classical lissencephaly have severe ID and develop intractable epilepsy. In addition they may present with early hypotonia which evolves in spasticity.<sup>1-3</sup>

The LIS1 protein plays a key role in cell migration of neuronal progenitors during brain development. LIS1 interacts with platelet-activating factor acetylhydrolase and cytoplasmic dynein. By binding to cytoplasmic dynein LIS1 regulates microtubule dependent cell motility. This process is disrupted in lissencephaly.<sup>2, 3, 5</sup> Neuronal cell migration is processed by nucleokinesis, which involves an extension of the neuron into which the nucleus is translocated via microtubule transport. It was shown that the cytoplasmic dynein 1 motor protein complex is the driving motor force of this process. Thus, LIS1 stimulates cytoplasmic dynein functions that involve neuronal migration and axon growth.<sup>2, 3, 5</sup>

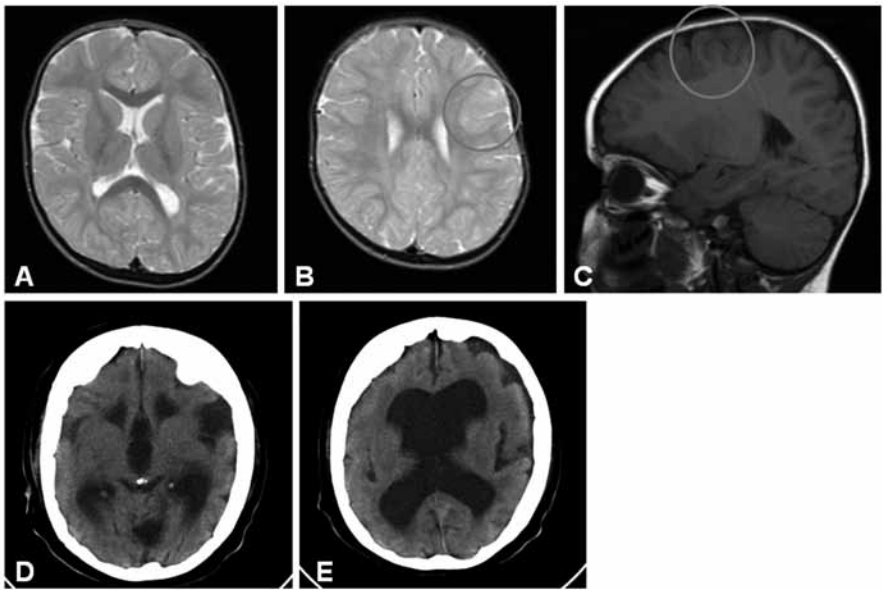
Recently, we reported a *de novo* missense mutation in *dynein, cytoplasmic 1, heavy chain 1 (DYNC1H1)* (MIM 600112)), a cytoplasmic dynein 1 motor complex gene, in a patient with ID as part of a larger study using a family-based exome sequencing approach.<sup>6</sup> Here we report the identification of a second *de novo* mutation in this gene in another patient with a distinct phenotype which overlaps with the patient of the previous study. These data together suggest that *de novo* mutations in *DYNC1H1* cause variable phenotypes including severe ID with variable neuronal migration defects, and peripheral neuropathy.

Patient 1 was previously reported by Vissers *et al.*<sup>6</sup> In brief, this boy was the first child born at term after an uneventful pregnancy with a weight of 3850g (60<sup>th</sup> centile) to healthy parents. At 6 months hypotonia was noted and physical therapy was started. Development was delayed. He could sit at 18 months and walk at 3 years. At the age of 4 years his developmental level was tested which showed a 2 years delay, consistent with moderately severe intellectual disability. At the age of 6 years and 6 months he could only speak several single words. On physical examination at the age of 2 years he had a normal height of 85.2 cm (15<sup>th</sup> centile) and head circumference of 47.5 cm (15<sup>th</sup> centile). He had mild (facial) dysmorphism consisting



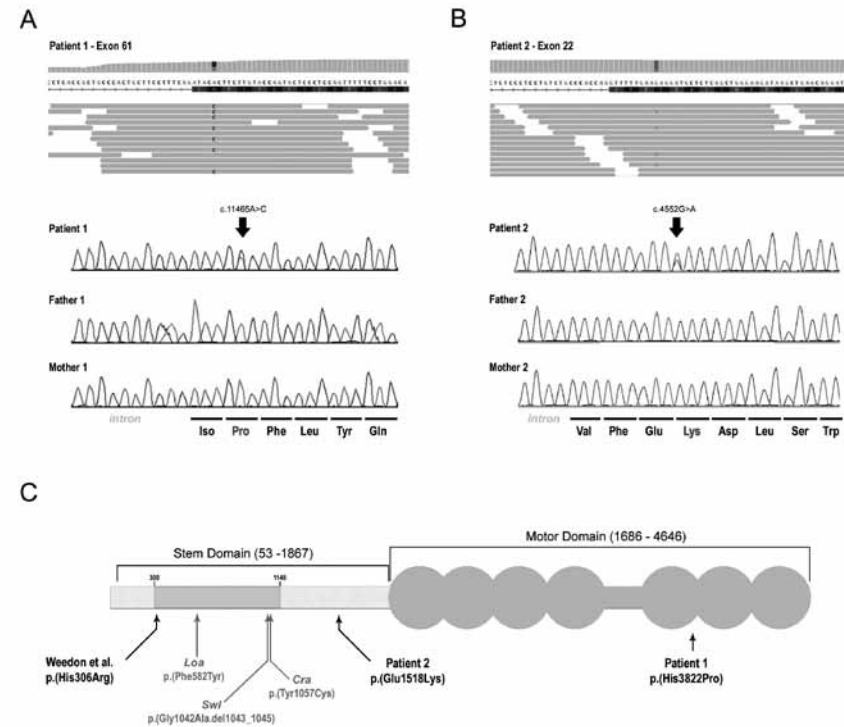
of prominent forehead, plagiocephalic skull, a hypotonic face with downslanted palpebral fissures and short broad hands and feet. On further physical examination at the age of 6 years and 6 months he had a normal height (122 cm (25-50<sup>th</sup> centile)) and head circumference (53.5 cm (84<sup>th</sup> centile)), and similar dysmorphism as on previous examinations. Abnormal neurologic findings included hypotonia, general reduced tendon reflexes and broad-based waddling gait with toe walking. Re-evaluation of the brain MRI that was performed at the age of 5 years and 6 months showed, despite its suboptimal quality, signs of bilateral cortical malformation with deficient gyration of the frontal lobes (without clear abnormalities of cortical thickness) and an area suggestive of focal cortical dysplasia (Figure 1 A-C). A family-based exome sequencing approach revealed a *de novo* c.11465A>C mutation in *DYNC1H1* (NM\_001376.4), predicting a p.(His3822Pro) substitution (Figure 2 A).<sup>6</sup> Although the Grantham score at 77 indicates a moderate physico-chemical difference, the PhyloP score at 5.12 suggests a very high conservation of

Figure 1 Cerebral imaging in patient 1 (A-C) en patient 2 (D-E)



Panels A-C show axial, T2 weighted (A and B) and sagittal, T1-weighted (C) MR images with deficient gyration of the frontal lobes and an area suspected for cortical dysplasia in patient 1 (circles in panels B and C). Figures D and E show CT images of patient 2, with enlarged ventricles, wide opercular regions, and clear signs of frontal lobe cortical malformation with an abnormal flat cortex with only a few, simple and shallow sulci.

Figure 2 Schematic representation of human DYNC1H1 and mutations in patient 1, 2 and the axonal (type 2) form of Charcot-Marie-Tooth (CMT2) disease family reported in Weedon *et al.*<sup>22</sup> together with the mutations of known mouse models.



Schematic representation of the mapped exome sequencing reads visualized using the *Integrative Genomics Viewer (IGV)* browser for patient 1 (panel A) and patient 2 (panel B), respectively. The upper part shows the per-base coverage, with coverage represented in gray indicating the wild-type base, whereas colored bases indicate the detection of variants. Also, a representation (part of) of exon 61 and exon 22 are provided for orientation. Individual sequence reads for patient 1 and patient 2 both show a heterozygous variant, which was followed up by Sanger sequencing, confirming the *de novo* occurrence in both patients. In panel (C), the DYNC1H1 protein is visualized according to Weedon *et al.* with the N-terminal region indicated by a gray horizontal bar, and the stem domain shown on above (amino acids 53-1867). The residues involving DYNC1H1 dimerisation (300-1140) are shown by a pink bar. The C-terminal motor domain (amino acids 1868-4646) is shown in purple colour, with the seven ATPase domains represented by circles and the stalk region by a horizontal bar. The equivalent positions of mutations in three mouse models, Loa, Swl, and Cra1, are shown below the representation. Note that the human protein contains two additional glycine residues at position 7 relative to mouse Dync1h1, that is numbering of equivalent residues in human DYNC1H1 is 2 higher than in mouse models. The three mutations reported in patients, including p.(His3822Pro) in patient 1, p.(Glu1518Lys) in patient 2, and p.(His306Arg) reported by Weedon *et al.* are indicated with arrows according to their relative positions of the functional domains of the DYNC1H1 protein.

the affected nucleotide among 46 vertebrate species. A prediction of the effect of this amino acid substitution at protein level by SIFT indicates that this mutation is deleterious to normal protein function.<sup>7</sup>

Patient 2 was a 51-year-old woman with severe ID. She had never been able to walk or speak. She was born after an uncomplicated pregnancy with a low-normal birth weight (5<sup>th</sup> centile). She had congenital clubfeet. At the age of 3 years she developed generalized epileptic seizures. At the age of 51 years she had a height of 135 cm (<0.6<sup>th</sup> centile) and a head circumference of 52 cm (2<sup>nd</sup> centile). She had small hands and feet (<3<sup>rd</sup> centile) and the toes were short and malpositioned. Craniofacial features included brachycephaly, prominent forehead, hypertelorism, deeply set eyes, wide mouth with everted lower lip and downturned corners of the mouth. She had secondary cataract as a result of automutilation, a kyphoscoliosis, spastic tetraplegia and was wheelchair dependent. At the age of 50 years she had developed progressive swallowing difficulties for which a gastrostomy tube was needed. A cerebral CT-scan at the age of 46 years, performed because of persistent seizures, showed wide lateral and third ventricles, and clear signs of cortical malformation with wide opercular regions and an abnormal flat cortex with only a few, simple and shallow sulci (especially in the frontal lobes) (Figure 1 D-E). It was not possible to obtain a cerebral MRI scan. Additional investigations comprising a routine screen in urine and blood for metabolic disorders including a screen for peroxisomal disorders and detailed chromosomal studies (250k SNP array), revealed no abnormalities. Also for this patient, a family-based exome sequencing approach was used and revealed a *de novo* c.4552G>A mutation in *DYNC1H1*, predicting a p.(Glu1518Lys) substitution (Figure 2 B). Similar to the mutation observed in patient 1 the Grantham score of 56 indicates a small physicochemical difference, whereas the PhyloP score at 6.03 indicates again a strong evolutionary conservation and SIFT predicts the mutation also to have a deleterious effect for protein function.

Examination of 445 exomes previously sequenced in our unit for unrelated medical conditions did not show either of the changes in *DYNC1H1* reported in patients 1 and 2. Moreover, in this entire cohort only 10 changes were seen in *DYNC1H1* that are not present in dbSNPv132. Of these, 9 variants are predicted to be synonymous changes, not affecting gene function, whereas the remaining variant predicts a missense p.Ser4603Ile substitution, which was detected in a single individual and involves a weakly conserved nucleotide (PhyloP -0.12). From this analysis, we conclude that nonsynonymous changes in *DYNC1H1* are very rare. Of note, retrospective analysis of genes known to cause neuronal migration disorders, including *LIS1*, *DCX*, *RELN*, *NDE1* and *TUBA1A*, did not reveal any pathogenic mutations whereas exon coverage for these genes was sufficient to detect such variants if present. Additionally, non-paternity and sample mix-up were excluded by

comparison of the patients 250k Affymetrix SNP profiles to their exome data (concordance >95%), and by the consistent segregation of uniquely inherited variants detected in the trios by exome sequencing. Thus, both changes represent *bona fide de novo* mutations in *DYNC1H1*. Combined with the fact that an average newborn is expected to have 0-2 nonsynonymous *de novo* mutations in his or her exome, the detection of nonsynonymous *de novo* events in *DYNC1H1* in two patients with similar phenotypes strongly suggests that the mutations are causal for the phenotype.

*Dynein, cytoplasmic 1, heavy chain 1 (DYNC1H1)* has 78 exons and encodes the heavy chain protein (DYNC1H1) of the cytoplasmic dynein 1 motor protein complex.<sup>8</sup> The cytoplasmic dynein 1 motor protein complex is composed of a homodimer of two heavy chains, encoded by *DYNC1H1*, and associated intermediate, light intermediate and light chains. This complex is involved in several cellular functions, including spindle pole organization and nuclear migration during mitosis, the minus end transport of cell organelles along microtubules, and retrograde axonal transport in neurons.<sup>8-11</sup> The C-terminal region of DYNC1H1 is the motor domain of the dynein complex and is arranged as a ring with six AAA (ATPases associated with cellular activities) domains, localizing ATP binding, required for energy generation, and a seventh uncharacterized domain, whereas the N-terminal region confines the stem domain, the region for homodimerization, and binding sites for the intermediate and light intermediate chains.<sup>8</sup> The cytoplasmic dynein 1 complex was formerly shown to play a key regulatory role in retrograde axonal transport in neurons, in which the heavy chains are responsible for movement along the microtubule.<sup>8-11</sup> Furthermore, several studies in model organisms provided evidence that the cytoplasmic dynein 1 complex directly interacts and coprecipitates with the LIS1/PAFAH1B protein.<sup>2, 3, 5, 12-15</sup> The dynein heavy chain protein and the intermediate chains biochemically interact with the WD-40 repeat region of LIS1. WD-40 repeats, also known as WD or beta-transducin repeats, are short ~40 amino acid motifs often terminating in a Trp-Asp (W-D) dipeptide, which act as a site for protein-protein interaction. Within the heavy chain, interactions with LIS1 are with the first AAA repeat, and the N-terminal cargo-binding region.<sup>2, 8, 12</sup>

The *Legs at odd angles (Loa/+)*, *Cramping I (Cral/+)* and *Sprawling (Swl/+)* mouse models, each harbour different missense mutations in the stem domain of *Dync1h1*. Their phenotypes include abnormal neuronal migration and axon growth, as well as neurodegeneration, caused by defective retrograde transport.<sup>11, 16-19</sup> Studies in these three mouse models in addition showed that heterozygous missense mutations in *Dync1h1* are associated with neurologic phenotypes including gait abnormalities, reduced muscle strength, reduced reflexes and abnormal proprioception and nociception in association with loss of motor and sensory

neurons.<sup>16-19</sup> *Loa* and *Cral* homozygous mice show a more severe phenotype and die within 24 hours after birth due to inability to feed and move.<sup>17</sup> Homozygous loss-of- function of *Dync1h1* is embryonically lethal, indicating an important role of *DYNC1H1* in human embryonic development.<sup>20</sup> Interestingly, *Loa* mice not only exhibit defects in retrograde axonal transport, but also show clear central migration defects in hippocampus and cortex that are similar to defects observed in reduced *LIS1* expression.<sup>21</sup> The clinical findings in patients 1 and 2 strongly overlap with the phenotype observed in the *Loa* mouse model and strengthen the previous functional links between *LIS1* and *DYNC1H1*.

Recently, a missense mutation c.917A>G, predicting a p.(His306Arg), was reported in a large pedigree with dominant axonal (type 2) Charcot-Marie-Tooth Disease (CMT2).<sup>22</sup> After comparison of the clinical presentation of our patients to those reported by Weedon *et al.*,<sup>22</sup> we noted some phenotypic overlap between patients 1 and 2 and the affected members of the CMT2 family (Table 1), though the overall presentation is importantly different.

The motor abnormalities and reduced tendon reflexes that were observed in patient 1, and the congenital clubfeet in patient 2, might indicate the presence of peripheral neuropathy similar to that reported in the CMT2 family. This remains speculative however, since neither formal neurophysiologic investigations nor peripheral nerve biopsy were performed in our patients.

Of note, in the CMT2 family, four of the 13 family members with a *DYNC1H1* mutation presented with global delay, speech delay and/or learning difficulties in addition to their motor problems. Whereas the delay in motor development may be explained by the peripheral neuropathy, the speech delay and/or learning difficulties obviously suggest additional involvement of the central nervous system. Brain imaging was not reported in the CMT2 family.

The important differences in phenotype between the two patients reported here and the dominant CMT2 family may indicate that heterozygous missense mutations in *DYNCH1* represent a broad phenotypic spectrum. Possibly, the presentation with severe ID and neuronal migration defects depends on the location and nature of the mutation (Figure 2 C). The mutation in the CMT2 family is located in the stem domain at the N- terminal end of the homodimerization region.<sup>22</sup> In patient 1 the mutation is located in the motor domain, where no previous mutations have been documented. The mutation of patient 2 is localized in the C-terminal end of the stem domain, but outside the homodimerization region (Figure 2 C). Further conclusions about genotype-phenotype correlations require that more patients with mutations in *DYNC1H1* are identified and carefully characterized at the clinical level.

**Table 1** Comparison of clinical features in patients 1 and 2 and the family reported by Weedon *et al.* with the axonal (type 2) form of Charcot-Marie-Tooth (CMT2) disease.<sup>22</sup>

	Patient 1 (age 5 years)	Patient 2 (age 51 years)	CMT2 family <sup>22</sup>
Clinical features			
ID/DD/LD	Severe ID	Severe ID	4/13 DD or LD
Neuronal migration defect	Mild	Severe	Not documented
Delayed motor milestones	Yes	Yes, never learnt to walk	9/13
Epilepsy	No	Yes	Not reported
Gait abnormalities (waddling and/or broad-based, frequent falls)	Yes	Cannot walk	5/13
Reduced reflexes	Yes	Not tested	7/13
Hypotonia and/or lordosis	Yes	No, hypertonia	13/13
Clubfeet	No	Yes	7/13

DD= global developmental delay; LD=learning difficulties and/or language delay

In summary, we conclude that *de novo* missense mutations in *DYNC1H1* are a novel cause of severe ID associated with variable neuronal migration defects. The phenotype may include peripheral neuropathy as well.

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**Competing interests**

No competing interest reported.

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### Contributors

The study was designed and the results were interpreted by M.H.W., T.Kl., M.A.A.P.W., L.E.L.M.V., H.G.B., H.v.B., B.C.J.H. and J.A.V. Subject ascertainment and recruitment was carried out by M.H.W., T.Kl., B.W.M.v.B. and B.B.A.d.V. Sequencing and genotyping was carried out and interpreted by L.E.L.M.V., T.Kr., J.d.L., D.L., J.S. and J.A.V. The manuscript was drafted by M.H.W., L.E.L.M.V., T.Kl. and J.A.V. All authors contributed to the final version of the paper.

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# 5.2

## Diagnostic Exome Sequencing in Patients with Severe Intellectual Disability

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## Abstract

Intellectual disability (ID) carries lifelong personal and social impact, but its causes remain largely unknown due to extensive clinical and genetic heterogeneity. We used family-based exome sequencing to detect causative mutations in a series of 100 patients with unexplained severe ID (IQ <50). Patients were previously clinically evaluated to exclude known causes of ID. After informed consent was obtained, the coding regions of >21,000 genes were sequenced in 100 patients and their unaffected parents. A data analysis pipeline was developed to identify and classify *de novo*, autosomal recessive and X-linked mutations. In addition, a high-throughput re-sequencing strategy was used to confirm new candidate ID genes in >750 ID patients. All mutations were evaluated by molecular genetic scientists and clinicians in the context of the patients' clinical presentation. Seventy-nine *de novo* mutations were identified in 53 of 100 patients. Predicted damaging *de novo* (n=10) and X-linked maternally-inherited (n=3) mutations in known ID genes yielded a diagnosis in 13 patients. Potentially causative *de novo* mutations in novel candidate ID genes were detected in 22 patients. For three of these candidate genes, additional *de novo* mutations were identified in patients with similar phenotypes, confirming that they are true ID genes. No causative autosomal recessive mutations were detected in this series. Thus, the total diagnostic yield was 16%, mostly comprising *de novo* mutations. *De novo* mutations represent an important cause of ID and exome sequencing is an effective diagnostic strategy for their detection.

## Introduction

Severe intellectual disability (ID), also referred to as cognitive impairment or mental retardation, affects approximately 0.5% of the population in Western countries<sup>1, 2</sup> and represents an important health burden. A clinical diagnosis of severe ID is generally based on an IQ below 50 and significant limitations in daily life functions. In early childhood, the diagnosis is based on significant developmental delays, including motor, cognitive and speech delays. Non-syndromic forms of ID are clinically indistinguishable due to absence of specific associated abnormalities. ID can be caused by non-genetic factors such as infections and perinatal asphyxia. In the developed world, however, most severe forms of ID are thought to have a genetic cause.<sup>2</sup> Genetic defects that cause ID range from large cytogenetically visible chromosomal abnormalities to point mutations in genes, but in spite of many years of genetic research and technological advances, the aetiology remains elusive in 55-60% of patients.<sup>3, 4</sup> Understanding the genetic cause of ID is of great benefit for patients and their families, as a diagnosis provides information on the progress and prognosis of the condition, precludes further unnecessary (invasive) tests, and may lead to appropriate therapy. Moreover, a conclusive diagnosis often facilitates access to appropriate medical and social supportive care.<sup>5-8</sup> Family members may benefit from knowledge on recurrence risk, availability of reproductive counselling and possible prenatal diagnosis.

Previously, we<sup>9</sup> and others<sup>10</sup> provided evidence for the hypothesis that rare *de novo* point mutations can be a major cause of severe ID. Recent studies have indicated that there are more *de novo* mutations than in healthy controls, highlighting the clinical importance of these mutations.<sup>11-15</sup> Additional support for the *de novo* hypothesis is found in the frequent sporadic occurrence of ID, without obvious environmental or familial factors. It has been estimated that mutations in well over 1,000 different genes may cause ID.<sup>16</sup> Because of this large mutational target, rare *de novo* mutations in these genes may collectively account for a considerable proportion of ID, thereby compensating for the severely reduced reproduction of patients with ID, and keeping its incidence in the general population stable.<sup>15</sup> In the absence of diagnostic clues from the clinical phenotype, unbiased whole genome or whole exome sequencing approaches are required to diagnose these genetic mutations causing ID.<sup>9, 17, 18</sup>

Here, we evaluate the role of *de novo* as well as X-linked and autosomal recessive inherited mutations in a series of 100 patients with unexplained ID (IQ <50) using a family-based exome sequencing approach in a clinical diagnostic setting. Previous extensive clinical and genetic evaluation of these patients had not led to an aetiological diagnosis. This series of patients therefore represents the end point of current diagnostic strategies where all conventional genetic resources have been

exhausted. It has been shown that this situation is the final conclusion for most patients with severe ID.<sup>19</sup> Exome sequencing revealed 79 *de novo* mutations in 53 patients, together with a limited number of X-linked and autosomal recessive inherited mutations. Careful analysis allowed us to provide a conclusive genetic diagnosis for 16% of ID patients. We conclude that exome sequencing offers an attractive diagnostic strategy for patients with severe ID of unknown cause.

Methods

Participants

We studied 100 patients (53 females, 47 males) with unexplained severe ID (IQ<50) and their unaffected parents (trios) (Table 1). This series is broadly representative for patients with severe ID referred to our tertiary clinic. All patients were clinically evaluated by a clinical geneticist (MHW, BWMvB, TKI, DAK, HGB, BBAdV). Prior to inclusion, patients had received an extensive diagnostic work-up including a genomic profile (250K Affymetrix SNP array), targeted gene tests and metabolic screening whenever indicated, but these had not led to a diagnosis. The study was approved by the Medical Ethics Committee of the Radboud University Medical Centre in Nijmegen, The Netherlands (NL13636.091.07; NL36191.091.11; NL19921.091.07), and we obtained written informed consent from the parents of all participants in the study. All experiments were performed at a diagnostic laboratory accredited to the CCKL Code of Practice, which is based on the EN/ISO 15189 (2003; Registration numbers R114/R115, Accreditation numbers 095/103).

Detection of mutations

Genomic DNA was isolated from blood with the use of a QIAgen DNA mini kit (QIAgen). Exomes were enriched using a SOLiD-optimized SureSelect human exome kit (Agilent v2, 50Mb), followed by SOLiD™4 sequencing (Life Technologies). After sequencing the trio, candidate *de novo* mutations were selected by excluding variants inherited from either parent, whereas recessive and X-linked mutations were identified by segregation analysis (Figure 1). Candidate *de novo* mutations were validated by conventional Sanger sequencing methods in DNAs of the patient and parents.

Testing for additional mutations in candidate ID genes

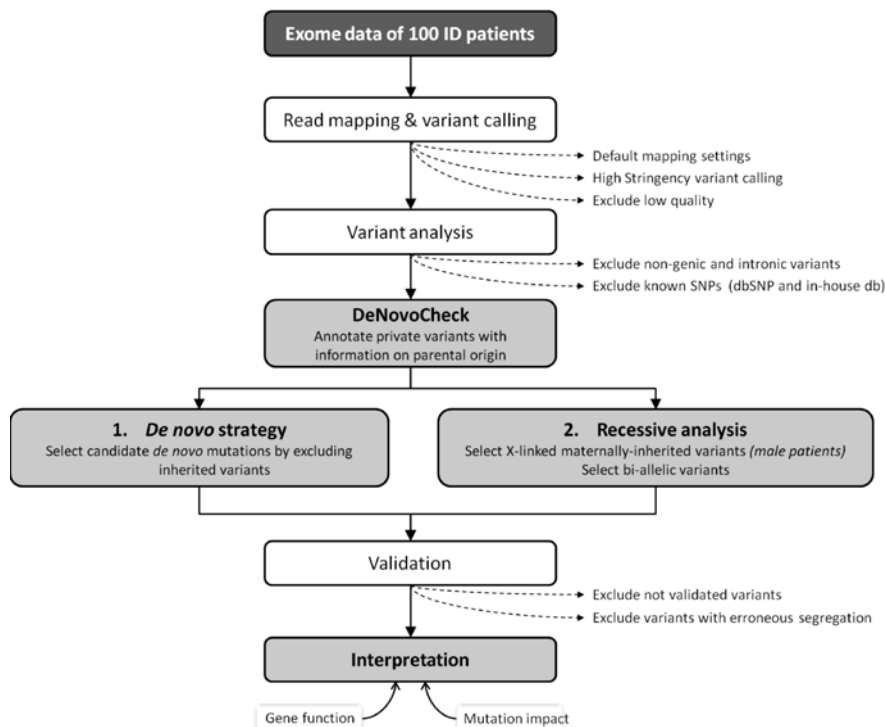
Five candidate ID genes (*DYNC1H1*, *KIF5C*, *ASH1L*, *GATAD2B* and *CTNNB1*) were re-sequenced using array-based enrichment on pooled DNAs from a second series of 765 ID patients (Table 2) selected from our in-house collection of 5,621 patients with undiagnosed ID. Written informed consent was previously obtained.

Table 1 Overview clinical details 100 ID patients.	
Level of ID	Number of patients
IQ <30	62
IQ 30-50	38
IQ 50-70	0
Gender	
Male	47
Female	53
Age groups	
<10 yrs	37
10-20 yrs	41
>20 yrs	22
Sibship size	
1	12
2	47
3	36
4	1
≥5	2
unknown	2
Number of major congenital anomalies	
0	62
1	31
2	7
3	0
Short stature	
Yes	24
No	76
Microcephaly	
Yes	30
No	70
Macrocephaly	
Yes	4
No	96
Epilepsy	
Yes	52
No	48

Table 1 Continued.

Abnormalities on brain imaging	
Yes	30
No	40
Not assessed	30
Cardiac malformation	
Yes	2
No	98
Abnormality of the urogenitary system	
Yes	13
No	87

Figure 1 Workflow Exome sequencing.



Experimental workflow for detecting and prioritizing sequence variants

Table 2 Clinical characteristics of ID patients selected to test for recurrent mutations in candidate ID genes.

Gender	
Male	511 (59.2%)
Female	352 (40.8%)
Age group	
< 10 yrs	415 (48.1%)
10-20 yrs	191 (22.1%)
> 20 yrs	257 (29.8%)
Age (years)	
Median age of series	11
Range	0-89
Interquartile Range	5-27.5
Level of ID	
IQ < 30	207 (24.0%)
IQ 30-50	145 (16.8%)
IQ 50-70	264 (30.6%)
not assessed*	247 (28.6%)
Clinical features	
Short stature	185 (21.4%)
Microcephaly	136 (15.8%)
Macrocephaly	30 (3.5%)
Epilepsy	154 (17.8%)
Structural CNS abnormalities	68 (7.9%)
Cardiac malformations	43 (5.0%)
Urogenital abnormalities	94 (10.9%)

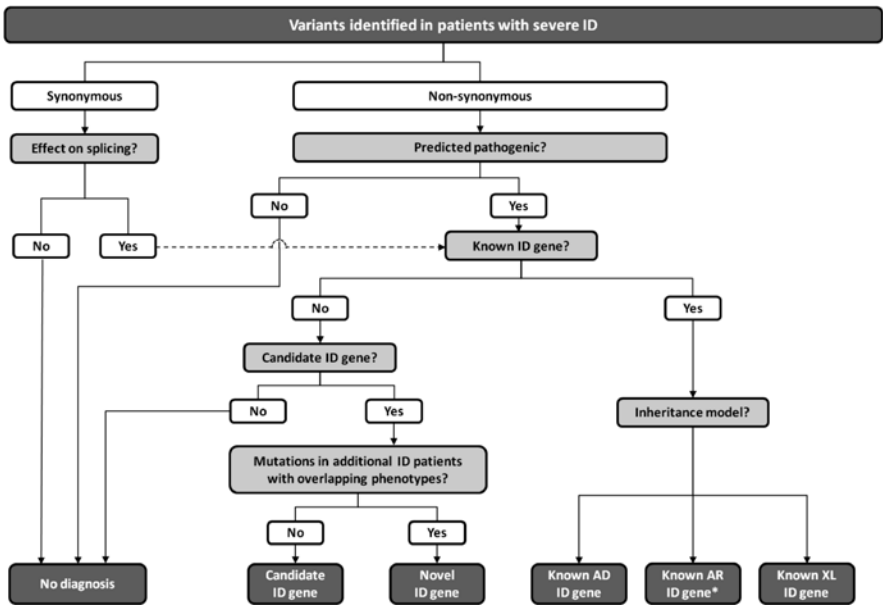
\*due to young age of patient

All patients were clinically evaluated by a clinical geneticist to exclude known causes of ID and genomic array analysis had not revealed causal copy number variants. Detected variants were annotated and prioritized according to their presumed relevance to disease. Variants fulfilling prioritization criteria were validated using conventional Sanger sequencing.

Interpretation of confirmed mutations and diagnostic reporting

Mutations were classified based on the existing guidelines for evaluation of variant pathogenicity<sup>20, 21</sup>, which included an evaluation of seven parameters including 1) gene function, 2) *in silico* prediction of mutation severity, 3) prediction of the functional effect at amino acid level, 4) evolutionary conservation, 5) brain expression patterns, 6) GO-term analysis, and 7) animal models, if available. Using a classification scheme (Figure 2), predicted pathogenic mutations in known and/or novel ID genes were reported as a highly likely cause of ID, if the patients showed matching phenotypes. Predicted pathogenic mutations in candidate ID genes were reported as a possible cause of ID. For patients without causal *de novo* or bi-allelic inherited mutations, the diagnostic report stated that the genetic cause of ID was not identified.

**Figure 2** Flow diagram for the classification of variants detected in patients with severe ID.



Results

Family-based exome sequencing of 100 patients with ID to identify *de novo* mutations

The power of family-based exome sequencing to provide a genetic diagnosis was evaluated for 100 patients with unexplained ID (IQ <50). An average of 24,324 genetic variants was detected per patient. An automated prioritization scheme was applied to systematically identify candidate *de novo* mutations (Figure 1), resulting in a total of 690 candidate *de novo* mutations (average of 7 per patient; range 2-20). Sanger sequencing confirmed the presence of 79 *de novo* mutations in 53 patients (range 1-4 per patient).

Description of *de novo* mutations and genes affected

Of 79 *de novo* mutations (affecting 77 genes), 16 mutations are predicted to lead to a synonymous change (Figure 2) of which none is predicted to alter splicing. Therefore, these mutations were classified as not causative for ID. The remaining 63 changes were non-synonymous and included 15 severely disruptive mutations (4 nonsense, 2 canonical splice site and 9 insertion-deletions) and 48 missense mutations. Based on random mutation modeling<sup>22</sup>, our observation of 4 nonsense mutations (6.1%) exceeded expectation (3.3%) suggestive for an elevated level of such mutations in ID. Moreover, PolyPhen-2 prediction of all 48 missense mutations showed a significant increase of probably damaging mutations. Twelve *de novo* mutations were detected in known ID genes, including six severely disruptive mutations and six missense mutations (Table 3). Three *de novo* mutations were detected in genes known to cause a recessive form of ID. These mutations would only be considered causal for ID if a second, inherited, predicted pathogenic mutations was identified. For *ARFGEF2* and *TUSC3* no second mutation was identified. For *LRP2* (patient 81), however, a rare paternally-inherited, predicted pathogenic variant was identified (c.6160G>A; p.(Asp2054Asn)). Analyses to show that the *de novo* event occurred on the maternal haplotype were inconclusive. Recessive *LRP2* mutations cause Donnai-Barrow syndrome and clinical re-evaluation of patient 81 confirmed this diagnosis. The remaining 51 *de novo* mutations were systematically analyzed for by gene function and predicted mutation impact for involvement in ID. This analysis identified 25 mutations in 24 candidate ID genes (Table 3). Interestingly for *DYNC1H1*, the patient enrolled in this study represented the second patient with a *de novo* mutation in this gene (for first patient see ref. 9). Phenotypic comparison of both patients showed overlap for severe ID and a variable neuronal migration defect.<sup>23</sup> (see Chapter 5.1).

**Table 3** Genes affected by *de novo* mutations with link to ID.

Type of mutation	Known ID genes	Novel ID genes <sup>#</sup>	Candidate ID genes	
Missense	<i>GRIN2A</i> <sup>¥</sup>	<i>DYNC1H1</i>	<i>GRIA1</i>	<i>PROX2</i>
	<i>GRIN2B</i>		<i>CAMKII<math>\beta</math></i>	<i>TANC2</i>
	<i>TCF4</i>		<i>KIF5C</i>	<i>TNPO2</i>
	<i>TUSC3</i> <sup>§</sup>		<i>COL4A3BP</i>	<i>PPP2R5D</i>
	<i>ARFGEF2</i> <sup>§</sup>		<i>RAPGEF1</i>	<i>ASH1L</i>
			<i>PHACTR1</i>	<i>LRP1</i>
			<i>PSMA7</i>	<i>MIB1</i>
			<i>EEF1A2</i>	<i>TRIO</i> <sup>*</sup>
Nonsense	<i>SCN2A</i>	<i>GATAD2B</i>	<i>PHIP</i>	<i>WAC</i>
Frameshift	<i>LRP2</i> <sup>*</sup>	<i>CTNNB1</i>	<i>ZMYM6</i>	
	<i>PDHA1</i>		<i>MTF1</i>	
	<i>TUBA1A</i>			
	<i>SLC6A8</i>			
Splice site	<i>SYNGAP1</i>		<i>MYT1L</i>	

¥: *De novo* mutations in two independent patients; §: Autosomal recessive ID gene where no second mutation was detected; \*: Autosomal recessive ID gene where a second rare-inherited mutation was detected; #: Based on additional *de novo* mutations in patients with phenotypic overlap.

Identification of novel ID genes by screening additional ID patients for *de novo* mutations

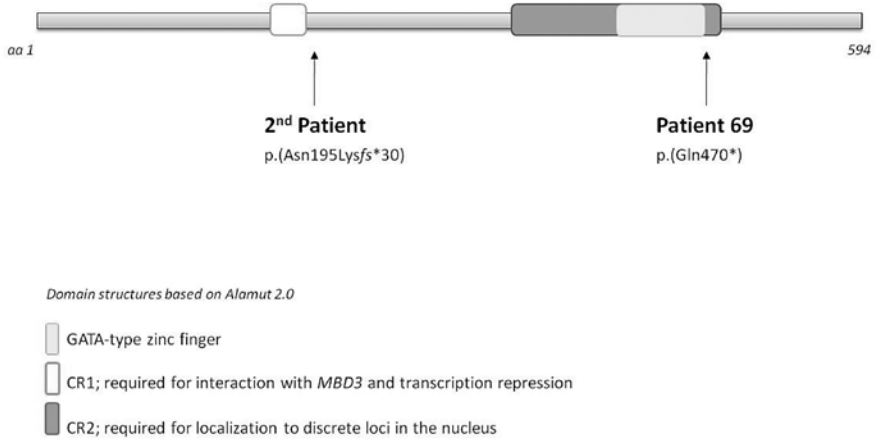
To provide further evidence for pathogenicity of *de novo* mutations in candidate ID genes, we re-analyzed previously generated exome data of undiagnosed patients with severe ID<sup>9</sup> and performed a targeted high-throughput sequencing study for five genes (*DYNC1H1*, *GATAD2B*, *ASH1L*, *KIF5C* and *CTNNB1*) to identify additional mutations in a second series of 765 ID patients (Table 4).

One additional *de novo* mutation was identified in the transcriptional repressor *GATAD2B*. Both *de novo* mutations observed in this gene, p.(Gln470\*) and p.(Asn195Lysfs\*30), are severely disruptive and predicted to lead to a loss-of-function (Figure 3). Both patients presented with severe cognitive and motor delays, limited speech and similar facial features. One additional severely disruptive *de novo* mutation was detected in *CTNNB1* (Figure 4). Both mutations, p.(Arg515\*) and

**Table 4** Variants validated in recurrence test.

	Total variants selected for Sanger validation	Confirmed
<i>KIF5C</i>	2	1
<i>GATAD2B</i>	4	2
<i>DYNC1H1</i>	32	8
<i>CTNNB1</i>	4	2
<i>ASH1L</i>	11	6
Total #	53	19 (36%)

**Figure 3** Schematic overview of *GATAD2B* mutations.

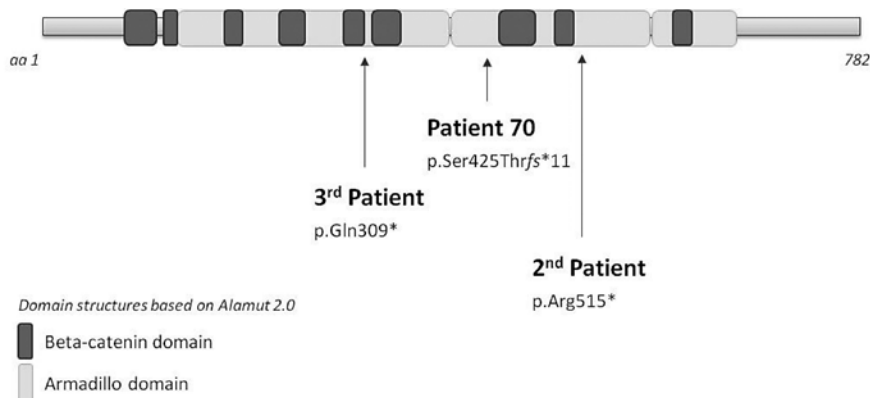


Schematic representation of *GATAD2B*, with its domain structure based on Alamut2.0. In addition, the mutations identified in this gene are indicated with their respective location at protein level. aa denotes amino acid number.

**Clinical comparison of patients with mutations in *GATAD2B*** – Two patients were shown to have a *de novo* mutation, p.(Gln470\*) and p.(Asn195Lysfs\*30) respectively, in the transcriptional repressor *GATAD2B*. Phenotypic comparison of these two patients was highly suggestive for a related genetic cause. They both had severe developmental delay with delayed motor milestones, only limited speech and overlapping facial features.



**Figure 4** Schematic overview of CTNNB1 mutations.



Schematic representation of CTNNB1, with its domain structure based on Alamut2.0. In addition, the mutations identified in this gene are indicated with their respective location at protein level. aa denotes amino acid number

**Clinical comparison of patients with mutations in CTNNB1** – Two patients were shown to have a *de novo* loss-of-function mutation in CTNNB1, including a p.(Arg515\*) nonsense mutation and a p.(Ser425Thrfs\*11) frameshift mutation. For a third patient, carrying a p.(Gln309\*) nonsense mutation, only maternal DNA was available for testing its segregation and was found to be absent. All patients presented with similar features comprising severe ID with absent or very limited speech, microcephaly and spasticity due to which the ability to walk was severely impaired.

p.(Ser425Thrfs\*11), are predicted to result in loss-of-function. A third patient carried a p.(Gln309\*) mutation in CTNNB1. This mutation was not present in maternal DNA, but paternal DNA was unavailable. All three patients presented with severe ID, absent or very limited speech, microcephaly and spasticity with severely impaired ability to walk .

Patients 4 and 15 showed *de novo* missense mutations in TRIO, p.(Asp1368Val) and p.(Thr2563Met) respectively. TRIO plays a role in several signaling pathways that control cell proliferation.<sup>24</sup> Phenotypic comparison revealed no similarities other than ID. Importantly, both patients also carried a *de novo* mutation in a known ID gene, ARHGEF9 and TCF4, respectively. Their phenotypes overlapped markedly with other patients showing mutations in ARHGEF9 and TCF4, indicating that these mutations are the most likely cause of ID, although the mutations in TRIO may still contribute.

### Identification of inherited mutations in autosomal recessive and X-linked genes

In 12 male patients, 14 X-linked inherited mutations were detected. Three of these mutations were located in known X-linked ID genes (PDHA1 and ARHGEF9 (n=2)). These were predicted to be pathogenic and the phenotypes observed in the patients matched that of those reported previously. In addition, 11 X-linked inherited mutations were identified in genes not previously associated with ID, of which one, TRPC5, was classified possibly causal. The analysis for autosomal recessive causes of ID revealed bi-allelic inherited mutations in nine genes, including two genes previously associated with an autosomal recessive form of ID (PCNT and VPS13B). None of these mutations were classified as possible cause for ID.

### Diagnostic implication of family-based exome sequencing in ID patients

Conclusive genetic diagnoses were obtained for ten patients with *de novo* mutations in known autosomal ID genes and for three male patients with severely disruptive maternally-inherited mutations in known X-linked ID genes (Table 5). Importantly, the phenotypes of these patients fit well with the phenotypes known for patients with mutations in these genes. No diagnostically relevant autosomal recessive mutations were identified. Thus, a diagnostic yield of 13% was obtained by mutations in known ID genes (Table 5).

**Table 5** Diagnostic yield of exome sequencing in patients with ID of unknown cause.

Positive diagnosis	Number of patients
<b>De novo mutations</b>	<b>13</b>
Autosomal dominant	10*
X-chromosomal	2
Autosomal recessive	1†
<b>Inherited mutations</b>	<b>3</b>
X-chromosomal	3
Autosomal recessive	0
<b>Total diagnostic yield</b>	<b>16</b>

\*: Seven mutations in previously known autosomal dominant ID genes, three in novel autosomal dominant ID genes; †: one *de novo* mutation and a second, inherited, predicted pathogenic mutation.

Table 6 Summary of diagnostic reports.

Patient	Number of <i>de novo</i> mutations	Molecular diagnosis for ID	Based on <i>[gene]</i> (inheritance)
1	0	NO	
2	1	Possible	<i>GRIA1 (de novo)</i>
3	0	NO	
4	1	Highly likely	<i>PDHA1 (X-linked maternally inherited)</i>
5	1	Possible	<i>PHIP (de novo)</i>
6	1	Possible	<i>PSMA7 (de novo)</i>
7	1	Highly likely	<i>TUBA1A (de novo)</i>
8	2	NO	
9	0	NO	
10	2	NO	
11	0	NO	
12	0	NO	
13	1	Highly likely	<i>SLC6A8 (de novo)</i>
14	0	NO	
15	4	Highly likely	<i>TCF4 (de novo)</i>
16	2	Highly likely	<i>SYNGAP1 (de novo)</i>
17	0	NO	
18	2	Highly likely	<i>ARHGEF9 (X-linked maternally inherited)</i>
19	0	NO	
20	0	NO	
21	1	NO	
22	2	Highly likely	<i>SCN2A (de novo)</i>
23	0	NO	
24	0	NO	
25	2	Highly likely	<i>GRIN2B (de novo)</i>
26	1	NO	
27	0	NO	
28	0	NO	
29	0	NO	
30	0	NO	
31	1	Possible	<i>PROX2 (de novo)</i>
32	2	Possible	<i>CAMK1G (de novo)</i>
33	0	NO	
34	0	NO	

Table 6 Continued.

Patient	Number of <i>de novo</i> mutations	Molecular diagnosis for ID	Based on <i>[gene]</i> (inheritance)
35	0	NO	
36	0	NO	
37	2	NO	
38	1	NO	
39	2	Possible	<i>LRP1 (de novo)</i>
40	2	Possible	<i>WAC / MIB1 (de novo)</i>
41	2	Highly likely	<i>ARHGEF9 (X-linked maternally inherited)</i>
42	1	Possible	<i>TANC2 (de novo)</i>
43	2	Possible	<i>TNPO2 (de novo)</i>
44	1	NO	
45	0	NO	
46	0	NO	
47	0	NO	
48	0	NO	
49	0	NO	
50	0	NO	
51	1	NO	
52	2	Highly likely	<i>GRIN2A (de novo)</i>
53	1	NO	
54	1	Highly likely	<i>DYNC1H1 (de novo)</i>
55	0	NO	
56	0	NO	
57	0	NO	
58	1	Highly likely	<i>GRIN2A (de novo)</i>
59	3	Highly likely	<i>PDHA1 (de novo)</i>
60	1	NO	
61	1	NO	
62	0	NO	
63	0	NO	
64	0	NO	
65	0	NO	
66	2	Possible	<i>ASH1L (de novo)</i>
67	0	NO	
68	1	NO	

Table 6 Continued.

Patient	Number of <i>de novo</i> mutations	Molecular diagnosis for ID	Based on [gene] (inheritance)
69	1	Highly likely	<i>GATAD2B</i> ( <i>de novo</i> )
70	3	Highly likely	<i>CTNNB1</i> ( <i>de novo</i> )
71	0	NO	
72	2	Possible	<i>PPP2R5D</i> ( <i>de novo</i> )
73	0	NO	
74	1	Possible	<i>KIF5C</i> ( <i>de novo</i> )
75	1	NO	
76	1	NO	
77	1	NO	
78	0	NO	
79	2	Possible	<i>COL4A3BP</i> ( <i>de novo</i> )
80	0	NO	
81	2	Highly likely	<i>LRP2</i> ( <i>de novo</i> + <i>rare inherited</i> )
82	1	NO	
83	0	NO	
84	0	NO	
85	0	NO	
86	1	NO	
87	2	Possible	<i>RAPGEF1</i> ( <i>de novo</i> )
88	1	Possible	<i>ZMYM6</i> ( <i>de novo</i> )
89	0	NO	
90	1	Possible	<i>PHACTR1</i> ( <i>de novo</i> )
91	2	Possible	<i>EEF1A2</i> ( <i>de novo</i> )
92	1	Possible	<i>MYT1L</i> ( <i>de novo</i> )
93	1	Possible	<i>MTF1</i> ( <i>de novo</i> ), <i>TRPC5</i> (X-linked maternally inherited)
94	0	NO	
95	0	NO	
96	0	NO	
97	1	NO	
98	0	NO	
99	0	NO	
100	0	NO	

Our study identified 24 novel candidate ID genes affected by *de novo* mutations. A pathogenic role for three of these genes was substantiated by the identification of additional ID patients with severely disruptive mutations. In each case, there was striking phenotypic overlap observed between the patients with mutations in the same gene. We therefore conclude that *DYNC1H1*, *GATAD2B* and *CTNNB1* are novel ID genes (Tables 3 and 5), raising the diagnostic yield to 16%.

Discussion

Mutations in over 400 genes have been linked to ID, but most of these mutations have a very low prevalence and their phenotypes are often indistinguishable. This argues for an unbiased diagnostic approach, especially since these 400 genes may still represent less than half of all ID genes. We implemented family-based diagnostic exome sequencing for patients with severe unexplained ID. Exome sequencing is a procedure that is highly amenable to automation. Variants with potential clinical consequences can easily be validated by Sanger sequencing as an independent method. No major hurdles were identified in the laboratory workflow in this study, allowing smooth integration of this process into diagnostics. *De novo* mutations were present in 53% of the patients and provided a conclusive genetic diagnosis in at least 13%, with an additional 3% of X-linked inherited mutations in known ID genes. This diagnostic yield is similar in magnitude but complementary to current genetic diagnostic chromosome analyses by genomic arrays.<sup>4, 25-27</sup> We expect that the diagnostic rate will increase further as additional patients with mutations in the novel candidate genes reported here will be identified. Identifying causal mutations in known ID genes in 16 of 100 patients provides clinically useful information for clinicians and families involved, as much is known about their prognosis and recurrence. The identification of the underlying genetic cause may also lead to specific treatment options or dietary advice. As an example, a ketogenic diet was advised for our patients with a *PDHA1* mutation.<sup>28</sup> In addition, a specific anti-epileptic treatment strategy, including the avoidance of sodium channel blockers, may be suggested for our patient with a *de novo* *SCN2A* mutation, as this leads to better seizure control and improvement of cognitive functioning and quality of life in patients with *SCN1A* mutations.<sup>29</sup> Our studies indicate that several of the new candidate ID genes that we identified will be confirmed as recurrently mutated in ID. We already identified additional *de novo* mutations in three of five genes tested (*DYNC1H1*, *GATAD2B* and *CTNNB1*) and detailed clinical analysis of these patients provided definitive evidence that these are novel ID genes. For *DYNC1H1* we identified a *de novo* mutation by exome sequencing both in our pilot study<sup>9</sup>, as well as in this series, and recently reported

the clinical overlap for these two cases.<sup>23</sup> The identification of recurrently mutated genes in combination with detailed clinical phenotyping is likely to rapidly reveal novel ID genes and identify specific clinical subtypes of ID that may require specific clinical management. No additional diagnoses were found when analyzing autosomal recessive inherited mutations, apart from *LRP2* which contained a *de novo* and a rare inherited mutation. The apparent absence of pathogenic mutations in autosomal recessive ID genes in our series indicates that this form of ID is rare in patients with isolated ID from non-consanguineous parents. Analysis of referrals for ID to our tertiary center shows that approximately 90% of patients with ID are from non-consanguineous parent and sporadic, 6% represent familial cases and 2% are sporadic cases born to consanguineous parents. X-linked forms of ID were diagnosed in five of the 100 patients, two of which occurred *de novo*. Mutations outside the coding regions as well as mosaic, digenic and/or oligogenic causes of ID remain to be defined.

Unbiased diagnostic approaches such as exome sequencing may also reveal clinically relevant mutations not related to the disease under investigation. An independent expert panel determined the clinical relevance of such incidental findings. Prior to enrolment in this study, all families were counselled about this possibility and consented to being informed if findings were deemed relevant by this panel. We note that not a single family objected to being informed about incidental findings. In this study, we encountered one incidental finding, a *de novo* c.517C>T (p.(Tyr173His)) change in *RB1*. Mutations in this gene are associated with retinoblastoma (RB; OMIM#180200), an embryonic malignant neoplasm of retinal origin, and associated with a low risk of developing osteosarcoma.<sup>30</sup> The expert panel considered the RB risk negligible for this patient as he already reached the age of 8 years, but decided that it was important to inform the parents of the small chance that a sudden painful swelling of the extremities could be caused by an osteosarcoma and they should consult an oncologist at the first symptoms. No further incidental findings were encountered.

In conclusion, our study establishes exome sequencing as a relevant diagnostic procedure for patients with severe ID of unknown cause. The current diagnostic yield in this series is 16% and this may well increase as methods further improve and more ID genes are identified.

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# 5.3

## Involvement of the kinesin family members *KIF4A* and *KIF5C* in intellectual disability and synaptic function

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Abstract

Kinesin superfamily (*KIF*) genes encode various motor proteins that are supposed to have fundamental roles in brain functioning, development, survival and plasticity, by regulating the transport of cargoes along microtubules within the axons, dendrites and synapses of neurons. Studies in mouse models support the important functions of *KIF* genes in the nervous system. Reports that relate *KIF* genes to intellectual disability (ID) are rare. Of interest, previous studies suggested that many ID genes impinge on synaptic function, in particular on the balance between excitatory and inhibitory synaptic input, and as such contribute to the pathophysiology of ID. By applying next generation sequencing (NGS) we identified likely pathogenic mutations in the X-linked *KIF4A* gene and the autosomal *KIF5C* gene and suggest that these genes are novel players in ID. Four males from one family with a mutation in *KIF4A* (c.1489-8\_1490delins10; p.?- exon skipping) showed mild to moderate ID and epilepsy. A female patient with a *de novo* missense mutation in *KIF5C* (c.11465A>C; p.(Glu237Lys)) presented with severe ID, epilepsy and microcephaly. Furthermore, we found supporting evidence for the causality of the mutations by showing that knock-down of *KIF4A* and *KIF5C* in rat primary neurons altered the balance between excitatory and inhibitory synaptic efficacy leading to altered neuronal excitability. Our results let us suggest that mutations in *KIF4A* and *KIF5C* cause ID by tipping the balance between excitatory and inhibitory excitability at the synapse.

Kinesin superfamily proteins (KIFs) are motor proteins involved in the movement of various cargoes, including vesicles, organelles, protein complexes, mRNAs and chromosomes, along the microtubules.<sup>1-4</sup> KIF proteins act together with motor proteins from the dynein and myosin superfamilies. These molecular motors are supposed to have fundamental roles in several processes in the brain, including neuronal functioning, development, survival and plasticity, by regulating the anterograde and retrograde transport within the axons, dendrites and synapses of neurons.<sup>1-4</sup> Studies in mouse models support the essential functions of *KIF* genes in the development and functioning of the nervous system. Mice with homozygous knockout mutations in *Kif1a*, *1b*, *2a*, *3a*, *3b*, *4a*, *5a* and *5b* show various neurological phenotypes including structural brain anomalies, decreased brain size, loss of neurons, reduced rate of neuronal apoptosis and perinatal lethality due to neurological problems.<sup>4-12</sup> The embryonic lethality of knockout mice for *Kif5b*, *Kif3a* and *3b*, and *Kif2a* suggest that these *Kif* genes have an important function in general development as well.<sup>5, 7, 8, 13</sup> Overexpression of *Kif17* in mice resulted in enhanced spatial learning.<sup>14</sup> Several KIF genes have previously been implicated in the pathogenesis of neuro-degenerative and neurodevelopmental disorders in humans. An overview is shown in **Table 1**.

**Table 1** KIF genes implicated in neurodegenerative and neurodevelopmental diseases in humans.

KIF gene	Disease	Inheritance	Reference
<i>KIF1A</i> (MIM 601255)	Hereditary spastic paraplegia type 30 (MIM 610358)	AR	4, 15, 16
	Hereditary sensory neuropathy, type IIC (MIM 614213)	AR	
	Non-syndromic ID	AD	
<i>KIF1B</i> (MIM 605995)	Charcot-Marie-Tooth disease , type 2A1 (MIM 118210)	AD	11, 17
	Multiple Sclerosis (MIM 126200)	Susceptibility locus	
<i>KIF5A</i> (MIM 602821)	Hereditary spastic paraplegia type 10 (MIM 604187)	AD	4, 18
<i>KIF7</i> (MIM 611254)	Acrocallosal syndrome (MIM 200990)	AR	19-21
	Hydroletharus syndrome (MIM 614120)	AR	
	Joubert syndrome 12 (MIM 200990)	AR	
	Syndromic ID	AR	
<i>KIF21A</i> (MIM 608283)	Congenital ophthalmoplegia (MIM 135700)	AD	22

AR: autosomal recessive; AD: autosomal dominant

Reports that relate *KIF* genes to intellectual disability (ID) rare. Putoux *et al.* identified homozygous mutations in *KIF7* (MIM 611254) in patients with acrocallosal syndrome (MIM 200990) and hydrolethrus syndrome (MIM 614120)<sup>19</sup>, and homozygous mutations in the same gene were also found in Joubert syndrome 12 (MIM 200990).<sup>20</sup> In these ciliary disorders ID is part of the phenotype. Najmabadi *et al.* identified a homozygous missense mutation in *KIF7* in a consanguineous family with ID, clubfeet, cataract, hearing loss and midface hypoplasia, detected by targeted sequencing of exons from homozygous linkage intervals.<sup>21</sup> More recently the first dominant *de novo* missense mutation in a *KIF* gene (*KIF1A*) was found in a patient with non-syndromic ID.<sup>16</sup>

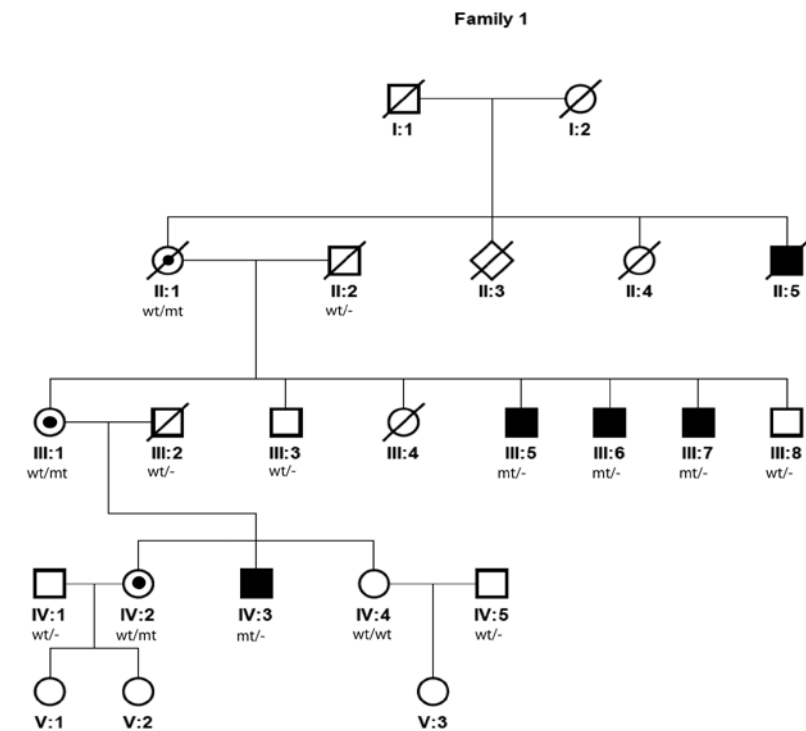
Here we report the identification of mutations in *KIF4A* (MIM 300521) and *KIF5C* (MIM 604593) in an X-linked ID family, presenting with mild to moderate ID and epilepsy, and in a sporadic patient with severe ID, microcephaly and epilepsy, respectively. In addition we studied the effects on synaptic function of these *KIF* genes using a knock-down system in rat primary neurons.

We searched for the causative mutation in a family (family 1) with five affected males in different generations (Figure 1), suggesting X-linked recessive inheritance. In the index patient we performed massive parallel sequencing after a targeted enrichment of all X-chromosome specific exons, as described by Kalscheuer *et al.*<sup>23</sup> The affected patients presented with mild to moderate ID, were able to speak simple sentences and lived and worked in sheltered places. Four of the five individuals had epilepsy that manifested at later childhood or adolescent age and comprised complex partial and generalized seizures (both absences and tonic-clonic seizures). Head circumferences were small to low-normal (0.6<sup>th</sup>-10<sup>th</sup> centile). Facial dysmorphisms were mild and non-specific. A CT-scan of the brain in two of the probands (III:7 at age 53 years and IV:3 at age 7 years) had shown central atrophy of both lateral hemispheres and wide posterior horns, respectively. Previous genetic investigations in this family included conventional karyotyping, genome-wide array analysis by the 2.7M Affymetrix array platform, DNA analysis of *FMR1* (MIM 309550) and *ARX* (MIM 300382), and a metabolic screen. The results did not provide an explanation for their phenotype. Linkage analysis indicated two intervals that could contain the pathogenic mutation, one of 70 cM on Xp22q13 and the other of 30 cM on Xq23q27, with a LOD-score of 1.8 (LOD-scores were calculated based on an X-linked recessive inheritance pattern with complete penetrance).

Targeted sequencing of the X-chromosome specific exons revealed a likely pathogenic in-frame mutation (c.1489-8\_1490delins10 ((p.? - exon skipping)) in *KIF4A* (NM\_012310.4). We subsequently confirmed the mutation by Sanger sequencing and found that it co-segregated with the phenotype in all affected males (a DNA sample of the uncle was not available). Three females from three generations were shown to be carriers of this mutation, but the mutation was absent

in two unaffected males (Figure 1). The mutation is located in the large Xp22q13 linkage interval. Further analysis of the effect on RNA expression showed an effect on splicing, including skipping of exon 15 (Figure 2).

**Figure 1** Pedigree of family 1.

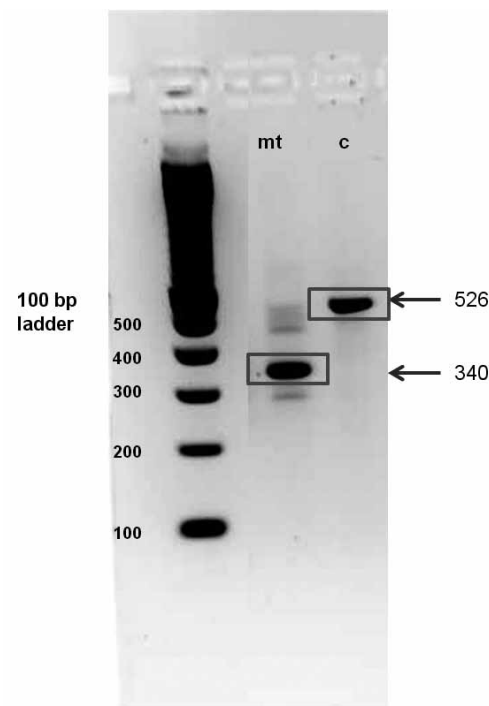


This figure shows the X-linked pedigree of family 1 with 5 affected males over 3 generations. The mutation in *KIF4A* was shown to co-segregate in affected males (III:5, III:6, III:7 and IV:3, a DNA sample of II:5 was not available) and was not present in two unaffected males (III:3 and III:8). Females II:1, III:1 and IV:2 were carriers.

This result let us suggest that *KIF4A* is a novel candidate XLID gene. *KIF4A* belongs to the class of the N5-kinesins and is highly expressed in differentiated young neurons. It consists of a N-terminal motor domain, a central stalk domain and a C-terminal C-domain.<sup>24</sup> Previous studies showed that *KIF4A* has various functions and plays a role in chromosome condensation and midzone spindle pole formation essential for cytokinesis and chromosome segregation during mitosis,<sup>9, 25-27</sup> as well



**Figure 2** RNA expression studies in cell-lines of patients from family 1 with a mutation in *KIF4A* and control cell-lines, showing effect on splicing.



This figure illustrates that the *KIF4* mutation affects the acceptor splice site of exon 15 leading to a skip of exon 15 (186 bp in length). RNA was isolated from a cell-line from an affected family member and a healthy control person. Primers for PCR analysis were designed at exon 13/14 and exon 16/17 boundaries and cDNA was synthesized. Lane 1 shows the band of the patient (**mt**), which is about 180 bp shorter in length as compared to the band in a healthy control (**c**), due to skipping of exon 15.

as in regulation of activity-dependent neuronal survival and apoptosis, thereby regulating the number of neurons during murine brain development.<sup>9</sup> After dissociation from an enzymatic protein called poly (ADP-ribose) polymerase 1 (PARP1), which prevents apoptosis, KIF4A moves into the neurites, where it possibly transports cargoes essential for neuronal development, such as the adhesion molecule L1 which is required for axon formation and synapse development.<sup>9, 28</sup> Although KIF4A was shown by several studies to be involved in spindle pole organization, most studies concluded that KIF4A deficient cells show a normal mitotic division. Aneuploidy analysis in cultured blood lymphocytes from one of the patients of

family 1 showed no increased level of aneuploid cells which is in agreement with the observation in most other studies that mitosis is not significantly disturbed.<sup>9, 25, 26</sup> Patient 2 was recently identified in a large family-based exome sequencing project performed by our group in a series of 100 patients with sporadic ID (see **chapter 5.2**).<sup>29</sup> In short, she was born after an uncomplicated pregnancy and delivery with normal growth parameters. She had a severe developmental delay, did not acquire the ability to speak and could only walk with support since the age of 9 -10 years. First seizures manifested at the age of 6 months. Her behavior was characterized by severe automutilation. During clinical evaluation at the age of 12 years she had a normal height (-1 SD), but a very dystrophic build and secondary microcephaly (head circumference: 49 cm (< -2.5 SD)). In addition she had small hands and feet. She had stereotypic hand movements and a slight hypertonia. There were no evident facial dysmorphisms. Because of the observed Angelman- and Rett-like syndrome phenotype, DNA analysis of *MECP2* (MIM 300005), *FOXG1* (MIM 164874), *CDKL5* (MIM 300203), *UBE3A* (MIM 601623) and 15q11q13 methylation tests were performed, which were all normal. Subsequent 250k SNP array analysis, DNA analysis of *ARX*, and a metabolic screen revealed no abnormalities as well. Family-based exome sequencing revealed a highly conserved (PhyloP 6.1) *de novo* c.11465A>C mutation in *KIF5C* (NM 004522.1), predicting a p.(Glu237Lys) substitution. The Grantham score of 56 indicated a moderate physicochemical difference.

*KIF5C* belongs to the class of N-1 kinesins together with *KIF5A* and *KIF5B*. *KIF5C* is specifically expressed in nervous tissue.<sup>1, 13, 30</sup> Mice with a homozygous knockout of *KIF5C* are viable and do not show gross abnormalities in the nervous system, except from a smaller brain size and relative loss of motor neurons to sensory neurons. This was explained by a high similarity between the three KIF5s and functional redundancy among these three KIF5s.<sup>13</sup> Deletion of *KIF5C* was reported in several patients with the 2q23.1 microdeletion syndrome.<sup>31, 32</sup> The mutation in *KIF5C* in the present patient 2 is a missense mutation affecting the motor domain of the KIF5C protein, and thus is in contrast to the other cases, not likely leading to haploinsufficiency. It might rather have a dominant negative effect resulting in abnormal heavy chain dimerization.

A growing body of work suggest that many ID genes impinge on synaptic function and as such contribute the pathophysiology of the disorder.<sup>33</sup> In particular the balance between excitatory and inhibitory synaptic input seems to be disturbed in common neurodevelopmental disorders including ID, autism and schizophrenia. Therefore, to find further supportive evidence that *KIF4A* and *KIF5C* are involved in the ID phenotypes of family 1 and patient 2 we performed functional studies at the level of synaptic function in hippocampal neurons.

Primary hippocampal neuron cultures were prepared from embryonic day 18 (E18) old rats as previously described.<sup>34</sup> Cultures were maintained in Neurobasal media

(GIBCO-Invitrogen) supplemented with B27, penicillin, streptomycin, and L-glutamine. Hippocampal neurons (6 DIV) were transfected with a mix of 3 siRNAs (5 nM) targeting *KIF4A* or *KIF5C*, and a fluorescently labeled scrambled siRNA using Lipofectamine 2000. All experiments were performed 3 days after transfection. The following validated siRNA's were obtained from Sigma Aldrich: *KIF4A*; SASI\_Rn02\_00290987 5'-CUAAUGACUUCUGUAGCUU-3'; SASI\_Rn02\_00290988 5'-CAUCUAGAACUGAAACCUA-3'; SASI\_Rn02\_00290989 5'-CAACCUAGAGGAAA-CAUUA-3'. *KIF5C*; SASI\_Rn02\_00231705 5'-GGUUACAAUGGGACAAUUU-3'; SASI\_Rn02\_00231706 5'-GCUUCUAGCUUCCACCAGA-3'; SASI\_Rn02\_00231707 5'-CAGAGAACUCCAGACUCUU-3'. The BLOCK-iT fluorescent oligo (Invitrogen) that is not homologous to any known genes was used as transfection efficiency detector and a negative control. Each siRNA was tested in hippocampal neurons and validated using quantitative PCR.

No reports are available on the synaptic function of *KIF4A*, whereas *KIF5* was recently reported to be involved in regulating both glutamatergic and GABAergic synaptic transmission in striatal and cortical neurons.<sup>35-37</sup> However the specific contribution of *KIF5C* has not been determined yet. To this end we examined the impact of *KIF4A* and *KIF5C* knockdown on both miniature excitatory, and inhibitory postsynaptic currents (mEPSC and mIPSC, respectively) in rat hippocampal neurons during a period of increased synaptogenesis, using validated siRNA's. Whole-cell recordings were obtained with Multiclamp 700B amplifiers (Axon Instruments). To monitor the effects of *KIF4A* and *KIF5C* knockdown on synaptic transmission, hippocampal cultures were transfected with siRNAs at 6DIV. Three days later, whole-cell recordings were obtained from transfected neurons under visual guidance using epifluorescence and transmitted light illumination. The recording chamber was perfused with artificial cerebrospinal fluid (ACSF) containing 119 mM NaCl, 2.5 mM KCl, 4 mM CaCl<sub>2</sub>, 4 mM MgCl<sub>2</sub>, 26 mM NaHCO<sub>3</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 11 mM glucose (pH 7.4), and gassed with 5% CO<sub>2</sub>/95% O<sub>2</sub>. Recordings were made at 30°C. Patch recording pipettes (3–5 MΩ) were filled with intracellular solution containing 115 mM cesium methanesulfonate, 20 mM CsCl, 10 mM HEPES, 2.5 mM MgCl<sub>2</sub>, 4 mM Na<sub>2</sub>ATP, 0.4 mM Na<sub>3</sub>GTP, 10 mM sodium phosphocreatine, and 0.6 mM EGTA (pH 7.25).

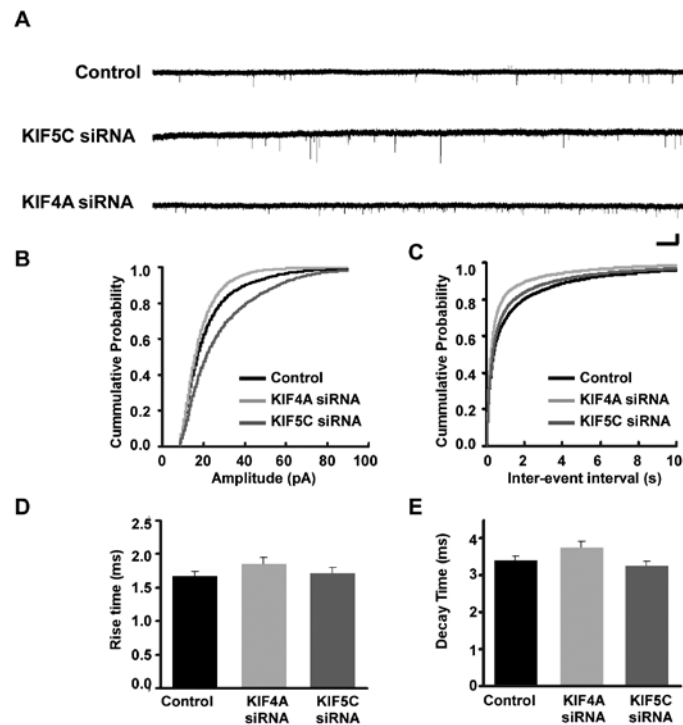
Spontaneous responses were recorded at –60 mV in ACSF containing 2.5 mM CaCl<sub>2</sub> and 1.2 mM MgCl<sub>2</sub> at 30°C. mEPSCs were recorded in the presence of 1 μM TTX and 0.1 mM picrotoxin, whereas mIPSCs were recorded in the presence of 10 μM CNQX and 100 μM APV. Five to 10 min of recordings were analyzed from each cell. Data were acquired at 5 kHz, filtered at 2 kHz, and analyzed using the Mini Analysis Program (Synaptosoft). All data are reported in a cumulative plot. Statistical significance was determined by the Kolmogorov-Smirnov test (KS-test). Significance was set to  $P < 0.01$ .

The amplitude of mEPSCs and mIPSCs is directly related to the postsynaptic strength, whereas the frequency is correlated to the presynaptic release properties and/or the amount of functional synapses. *KIF4A* knockdown resulted in a significant decrease in mEPSC amplitude and a significant increase in mEPSC frequency compared to control siRNA (KS-test,  $P < 0.001$ ), suggesting that the development of excitatory synapses is hampered in neurons lacking *KIF4A* (Figure 3A-C). In contrast, knockdown of *KIF5C* resulted in a significant increase in both amplitude and frequency of mEPSCs (Figure 3A-C, KS-test,  $P < 0.001$ ), indicating that loss of *KIF5C* promotes excitatory synapse formation. This is somehow surprising since interfering with all *KIF5* isoforms via the kinesin light chain was reported to decrease excitatory synaptic function.<sup>37</sup> This result is thus indicative for a specific function of *KIF5C* at excitatory synapses. For both *KIF4A* and *KIF5C*, we did not observe any changes in the kinetics of mEPSCs, indicating that the composition and/or function of the glutamate receptors mediating the mEPSCs, e.g. AMPARs, was unchanged (Figure 3D, E). Next we measured mIPSCs, a response from quantal release of single GABA<sub>A</sub> vesicles. Down-regulation of *KIF4A* decreased mIPSC frequency but not amplitude, indicating that *KIF4A* affects presynaptic GABA<sub>A</sub> release and /or inhibitory synapse formation without affecting the amount GABA<sub>A</sub>-receptors (GABAARs) at inhibitory synapses (Figure 4A-C). For *KIF5C* the effects on mIPSCs were much more pronounced. Loss of *KIF5C* showed a decrease in amplitude and frequency of mIPSC compared to control conditions (Figure 4A-C). This is in accordance with recent studies in which down-regulation of the *KIF5* family in cortical neurons resulted in a decreased inhibitory input by regulating the trafficking of GABAARs at inhibitory synapses.<sup>35, 37</sup> Knocking down *KIF4A* and *KIF5C* did not change the kinetics of mIPSCs indicating that they are not involved in the trafficking of a specific GABAAR subtype (Figure 4D, E). Together our data show that both *KIF4A* and *KIF5C* are important proteins that regulate synapse development. In particular *KIF5C* is a critical molecule in controlling the tight balance between excitatory and inhibitory inputs during development since knocking down *KIF5C* diminished inhibitory and increased excitatory synaptic efficacy. A disturbed balance between excitatory and inhibitory drive could explain the presence of epilepsy in the patients.

In summary, our studies support the involvement of the kinesin superfamily members genes *KIF4A* and *KIF5C* in the ID phenotypes of the presented patients. Identification of other patients with mutations in these genes should further confirm the role of *KIF4A* and *KIF5C* in ID phenotypes and may give further insights in the clinical spectrum. At the functional level we found that both *KIF4A* and *KIF5C* may contribute to the pathophysiology of ID by altering the balance between excitatory and inhibitory synaptic efficacy leading to changed neuronal excitability. As such



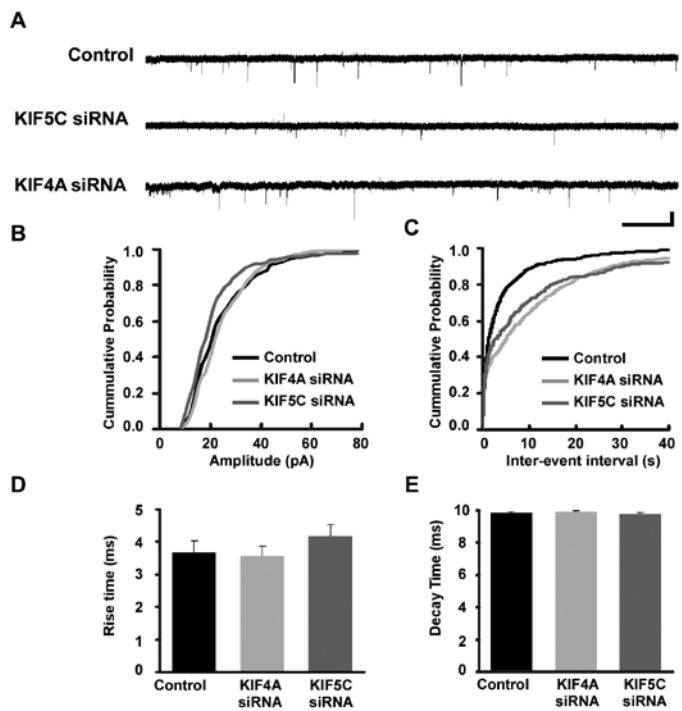
**Figure 3** Effect of KIF4A and KIF5C on mEPSC.



(A) Representative whole-cell current traces (mEPSC) of a control neuron or a neuron transfected with siRNAs against *KIF4A* or *KIF5C* at holding potential of  $-60$  mV. Scale bars: 1.2 s and 20 pA (B, C) Cumulative probability histograms of mEPSC amplitude and inter-event intervals in control ( $n=39$  neurons), *KIF4A* siRNA treated neurons ( $n=30$  neurons) and *KIF5C* siRNA treated neurons ( $n=34$  neurons). (D, E) Average rise (D) and decay time (E) of mEPSCs of control neurons and neurons treated with siRNAs targeting *KIF4A* or *KIF5C*. Differences between the conditions for the displayed mEPSC amplitude and inter-event interval cumulative distributions are statistically significant ( $P < 0.001$ , using Kolmogorov-Smirnov test). Error bars indicate S.E.M. Histograms show means + S.E.M.

they can be added to the growing list of ID genes that influence synaptic functioning.<sup>33</sup> Of interest, we recently reported the involvement of another component of the microtubule transport pathway in two patients with a similar ID phenotype. Both patients had a conserved *de novo* mutation in *dynein, cytoplasmic 1, heavy chain 1* (*DYNC1H1*, MIM 600112), encoding a component of the cytoplasmic dynein 1 motor protein complex.<sup>38</sup> Therefore, our present and previous findings indicate that the

**Figure 4** Effect of KIF4A and KIF5C on mIPSC.



(A) Representative whole-cell current traces (mIPSC) of a control neuron or a neuron transfected with siRNAs against *KIF4A* or *KIF5C* at holding potential of  $-60$  mV. Scale bars: 16 s and 25 pA (B, C) Cumulative probability histograms of mIPSC amplitude and inter-event intervals in control ( $n=7$  neurons), *KIF4A* siRNA treated neurons ( $n=10$  neurons) and *KIF5C* siRNA treated neurons ( $n=8$  neurons). (D, E) Average rise (D) and decay time (E) of mIPSCs of control neurons and neurons treated with siRNAs targeting *KIF4A* or *KIF5C*. Differences between control and KIF4A siRNA are statistically significant for the displayed inter-event interval cumulative distributions but not for the mIPSC amplitude. Differences between control and KIF5C siRNA are statistically significant for the displayed mIPSC amplitude and inter-event interval cumulative distributions ( $P < 0.001$ , using Kolmogorov-Smirnov test). Error bars indicate S.E.M. Histograms show means + S.E.M.

microtubule transport pathway may be an evolving ID pathway, and other genes encoding microtubule transport proteins may well be potential candidates for involvement in ID phenotypes as well.

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The authors declare no conflicts of interest.

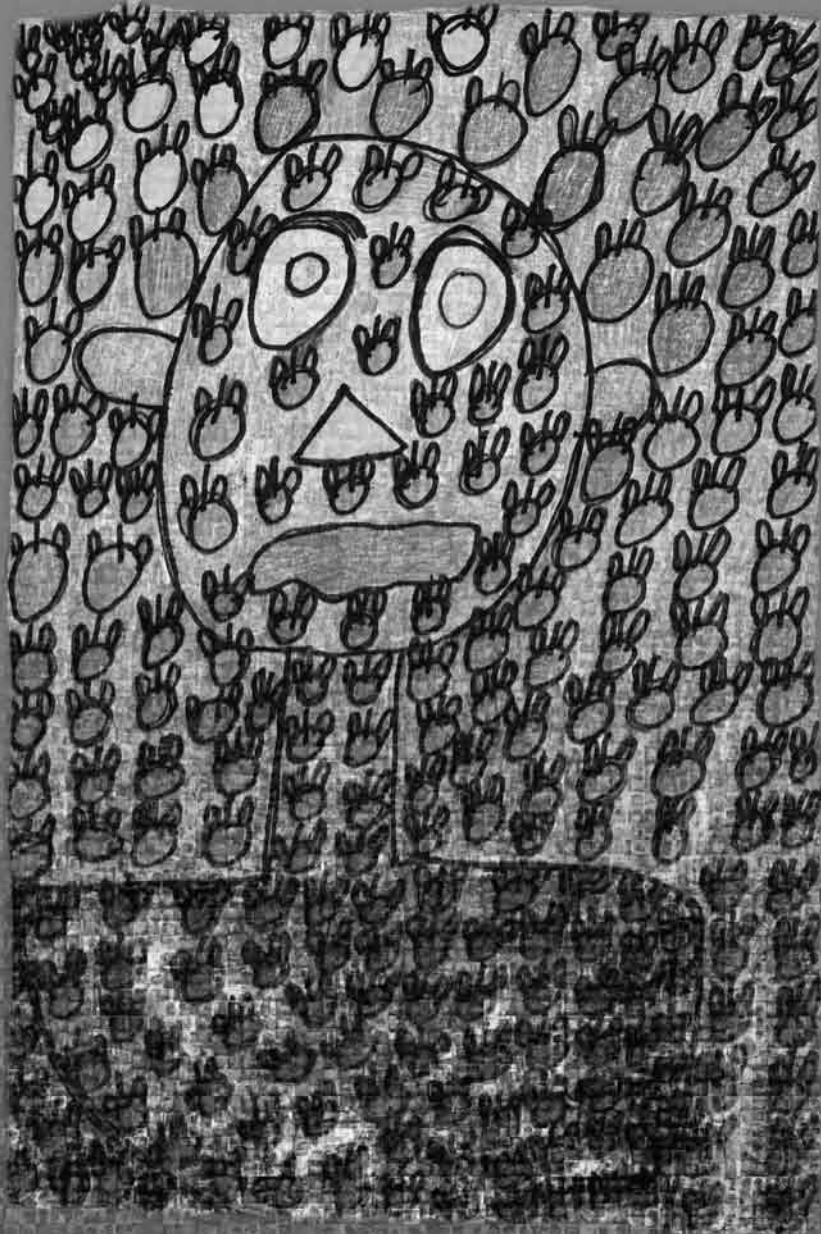
## Web resources

Online Mendelian Inheritance in Man (OMIM), <http://www.omim.org/>.

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# 6

## General overview of results

- 6.1 Diagnostic phase
- 6.2 Diagnostic related research phase
- 6.3 Total yield of diagnostic phase and diagnostic related research phase
- 6.4 Comparison between patients *with* and *without* a diagnosis

In **chapters 3-5**, various specific findings resulting from our studies were highlighted. This chapter describes the general overview of the results in the total study cohort, which is described in **chapter 2**.

6.1 Diagnostic phase

A general overview of the results in the diagnostic phase is shown in **Table 1**. In total, in 178 of 234 index patients (76.1%) the diagnostic tests revealed an abnormal finding. In 43 cases (18.4%) the identified variants were (likely) pathogenic and in 55 (23.5%) of the cases the clinical relevance of the findings was unknown. Criteria that were used to assess pathogenicity are described in **chapter 2** of this thesis. In the 18.4% of cases with a (likely) diagnosis, the majority (about 2/3) was explained by a copy number variation (CNV). Monogenic defects and metabolic abnormalities accounted for the other cases.

**Table 1** General overview of abnormal findings in the diagnostic phase.

Abnormal finding	(Likely) pathogenic <sup>a</sup>	Unknown <sup>a</sup>	(Likely) non-pathogenic <sup>a</sup>	Total N <sup>a</sup>
Chromosomal CNV	29 (12.4)	16 (6.8)	11 (4.7)	56 (23.9)
Monogenic defect	11 (4.7)	0 (0)	3 (1.3)	14 (6.0)
Homozygous region (>3 Mb)	0 <sup>b</sup> (0)	21 (9.0)	0 (0)	21 (9.0)
Metabolic abnormality	3 <sup>c</sup> (1.3)	18 (7.7)	66 (28.2)	87 (37.2)
Total N (%)	43 (18.4)	55 (23.5)	80 (34.2)	178 (76.1)

CNV: copy number variation  
<sup>a</sup> number (%) of 234 index participants  
<sup>b</sup> in 1 patient (09-0038) a 'pathogenic' homozygous region was found, which was explained by a mosaic trisomy. This patient was included in the chromosomal CNV group  
<sup>c</sup> all mitochondrial disorders, not otherwise specified, because further investigations (in skin biopsy) were not possible

(Likely) pathogenic findings

In 12.4% of all individuals a (likely) pathogenic chromosomal aberration was detected. A detailed overview is shown in **table 2**. (Likely) pathogenic chromosomal copy number variations (CNVs) included well-known microdeletion syndromes, such as 1qter microdeletion syndrome (OMIM 612337), Sotos syndrome (5q35



**Table 2** (Likely) pathogenic chromosomal copy number variations detected in the total cohort (N= 29).

Case number	Patient ID	Phenotype	Sex	CNV	Chromosomal region	Start-End Mb positions (Hg19)	Size/Gene content	Inheritance	Chromosomal analysis	References
1	08-0009	Sev. ID; D; MC; GR growth retardation; N; E	F	Loss	1qter	244.55-249.14	4.4 Mb/> 40 genes	<i>De novo</i>	250k SNP array analysis	<sup>16</sup>
2	09-0018	Mild ID; CA	M	Loss	1p21.3	97.55-98.96	1.41 Mb/3 genes	<i>De novo</i>	250k SNP array analysis	<sup>17</sup>
3	09-0044 09-0046 (sibs)	Mild ID; F; N	M	Loss	1p21.3	97.72-99.48	1.7 Mb/6 genes	Inherited (parents not tested)	250k SNP array analysis	<sup>17</sup>
4	11-0021	Mod.ID; D; CA; N	M	Gain	1q12q21.1	144.04-145.71	1.7 Mb/17 genes	Unknown (father not available)	250k SNP array analysis	<sup>18-20</sup> Decipher: Case numbers 250709 and 255915
5	10-0059	Mild ID, anxiety disorder	F	Loss	2p16.3	51.02-51.48	455kb/1 gene ( <i>NRXN1</i> )	<i>De novo</i>	2.7M array analysis	<sup>21, 22</sup> Decipher: Case numbers 951,250904, 251242
6	09-0068	Mod. ID; D; CA	M	Loss	2q37.3	242.01-242.36	350 kb/8 genes	NT (parents not available)	250k SNP array analysis	<sup>23</sup>
7	11-0028	Sev. ID; GR; D; E	M	loss	3p25.3	8.42-9.58	1.16 Mb/12 genes	NT (parents not available)	250k SNP array analysis	<sup>24</sup> Decipher: Case number 248715
8	09-0042	Sev.ID; GR; E MC; D	M	loss gain	3p26.3 14q31.1q32.33 (t(3;14))	0.73-1.66 81.83-107.29	1.6 Mb/3 genes ~25 Mb/> 50 genes	Maternal balanced translocation	250k SNP array analysis	<sup>25-28</sup>
9	08-0012	Sev. ID; GR; MC; Hypogonadism; Hearing loss; Behaviour problems	F	loss	3q24q25.4	143.14-159.39	16.2 Mb/> 30 genes	NT (parents not available)	Initially routine cytogenetic analysis by G-banding; Further delineation by 250k SNP array analysis	<sup>29</sup>
10	10-0042	Sev. ID; GR; Small hands and feet; E;D	F	loss	4q21.21q21.23	79.93-85.12	5.2 Mb/> 32 genes	<i>De novo</i>	2.7M array	<sup>30</sup> Decipher: Case numbers 252386, 994
11	09-0075	Sev. ID; MC; Behaviour problems; Kyphoscoliosis;	M	loss	5q35.2q35.3	175.57-176.89	1.3 Mb/~20 genes, including <i>NSD1</i> (Sotos syndrome)	<i>De novo</i>	250k SNP array analysis	<sup>31</sup>
12	10-0099	Mild ID; High myopia; GR; MC; Psychiatric problems	F	gain	7q36.1	151.79-152.50	710 kb/6 genes	NT (parents not available)	250k SNP array analysis	Decipher: Case number 255588 Case in personal database

Table 2 Continued.

Case number	Patient ID	Phenotype	Sex	CNV	Chromosomal region	Start-End Mb positions (Hg19)	Size/Gene content	Inheritance	Chromosomal analysis	References
13	08-0030	Sev. ID; MC; E; D; Depigmentation spots	M	loss	7q22.1	98.76-100.80	2 Mb/> 70 genes	Unknown (father not available)	250k SNP array analysis	Decipher: Case numbers 854, 256143, 248317, 253694, 252368. Ecaruca: case numbers 4328, 290, 2852, 2521, 1720
14	09-0038	Mild ID; IUGR; Cardiac arrhythmia; Kyphoscoliosis; Dolichocephaly	M	gain	Low grade mosaic trisomy 9, uniparental disomy 9	Large homozygous stretches chromosome 9	Total chromosome 9 (mosaic)	NT	250k SNP array FISH buccal swap: low mosaic pattern trisomy 9	<sup>32-34</sup>
15	11-0022	Mod. ID; CA;E	M	gain	12p11.23	27.29-27.79	500 kb/ 4 genes	<i>De novo</i>	250k SNP array	Decipher Case number 256785 Two overlapping cases with overlapping phenotype in our personal database (inherited)
16	09-0025	Sev. ID; MC; Small hands and feet; N; Behaviour problems; CA;E	F	loss	14q12	29.83-31.73	1.9 Mb/8 genes	<i>De novo</i>	250k SNP array	<sup>35-37</sup> Decipher: Case numbers 3810, 248405, 2060, 251718
17	09-0028	Mild ID, D; CA	M	gain	15q11.2-q15.1 (marker chromosome 15, 3 copies BP1-BP6)	22.75 – 35.21	12.7 Mb/> tens of genes	Maternal	250k SNP array Additional MLPA	<sup>38</sup>
18	09-0048	Mild ID; D	F	gain	15q11.2q13.1	23.70-28.53	4,8 Mb/ 19 genes	Unknown (parents not available)	250k SNP array Additional conventional karyotyping	<sup>39, 40</sup>
19	11-0033	Mild ID; Fa	M	gain	15q13.2q13.3	30.91-32.51	1.58 Mb/ 13 genes	Paternal	250 k SNP array	<sup>40</sup> Decipher: Multiple overlapping gains
20	10-0039	Mod.ID; D; CA	F	loss	17p11.2	16.76-20.43	3,7 Mb/tens of genes	<i>De novo</i>	2.7M array	OMIM 182290 (Smith-Magenis Syndrome)
21	09-0060	Sev. ID	M	loss	16q23.3q24.1	82.85-84.49	1,64 Mb/13 genes	Unknown (parents not available)	250k SNP array	-
22	10-0060	Mild ID; CA	M	gain	17q12	34.38-36.45	1.8 Mb/ 25 genes	maternal	250k SNP array	<sup>41</sup> Decipher: Case numbers 2843 and 1718

Table 2 Continued.

Case number	Patient ID	Phenotype	Sex	CNV	Chromosomal region	Start-End Mb positions (Hg19)	Size/Gene content	Inheritance	Chromosomal analysis	References
23	08-0025	Mod. ID; D; E	F	loss	17q21.31	43.74-44.24	460 kb/4 genes	De novo	250k SNP array	42
24	09-0049	Sev. ID; D; CA; GR; E	M	loss gain	20p13 20q13.33	0.60-1.12 59.95-62.38	1,06 Mb/21 genes 2,46 Mb/>50 genes	Inherited (balanced rearrangement in mother)	250k SNP array	43
25	10-0048	Sev.ID; D; N; E	M	loss	22q13.32q13.33	49.40-51.20	1.8 Mb/ tens of genes	Unknown (parents not available)	2.7M array	44, 45 OMIM 606232 (Phelan-Mc Dermid syndrome)
26	10-0055	Sev. ID; D; CA; Fa	M	Loss	22q13.32q13.33	49.03-51.18	2.12 Mb/ tens of genes	Inherited (identical deletion in affected brother)	250k SNP array	44, 45 OMIM 606232 (Phelan-Mc Dermid syndrome)
27	11-0008	Mild ID; D	M	gain	22q11.22q11.23	23.07-24.96	1.9 Mb/43 genes	Maternal (learning difficulties)	250k SNP array	46, 47 Decipher: Case numbers 251348, 2727
28	09-0085	Sev. ID; GR; D; CA	M	loss	Xp22.31p22.33	0.14-6.41	6.4 Mb/20 genes	Maternal (learning difficulties)	250k SNP array	48-50
29	09-0015	Mod. ID; N	M	loss	Xq22.2	102.958 – 102.977	19 kb/2 genes	maternal	Cytoscan HD array	51

NT= not tested. Sev./Mod./Mild ID: severe/moderate/mild ID; D: dysmorphism; MC: microcephaly; MaC: macrocephaly; (IU)GR: (intra-uterine) growth retardation/short stature; CA: congenital anomaly; Fa: ID in family; N: neurological features; E: epilepsy.

Classifications of findings in each individual patient in this cohort were based on careful consideration of the full picture of the criteria for assessment of clinical relevance as mentioned in **Chapter 2, Table 4**. None of the criteria was absolute and each criterion was considered in perspective to the other criteria, the currently available information in literature and databases, and the phenotype of the patient. Microdeletions and microduplications that can be considered as a strong risk factor for ID but have sporadically been reported in healthy controls or are inherited, such as 15q13 microdeletions and duplications, were either classified as (likely) pathogenic or as of unknown clinical significance depending on the phenotype of the respective patient.

microdeletion, OMIM 177550), Smith-Magenis syndrome (17p11.2 microdeletion, OMIM 182290), 15q13 microdeletion (OMIM 612002) and microduplication syndromes (OMIM 608636), 17q21.31 microdeletion syndrome (OMIM 610443) and 22q13 microdeletion syndrome (OMIM 606232) (case numbers 1, 11, 18-21, 23 and 24). In addition, several more rarely reported CNVs, and novel CNVs (case numbers 2 and 3) were included in the group of (likely) pathogenic CNVs.

In about 4.7% of the patients a monogenic defect was identified as the cause of ID. Monogenic defects are summarized in **table 3**.

In only three patients (1.3%) a pertinent metabolic cause of ID could be established, in all three cases a mitochondrial disorder. The mitochondrial defects could not be further specified, because the parents, the legal representatives and/or the patients themselves were reluctant to give permission for a skin or muscle biopsy for further investigations.

In the general overview of the yield of this cohort study (**table 1**) only molecularly confirmed genetic diagnoses were taken into account. For example, one patient had an evident clinical diagnosis of Gomez-Lopez-Hernandez syndrome (OMIM 601853), though the underlying genetic cause has not been elucidated. In contrast with most previous cohort studies<sup>1-6</sup>, we have not included such patients in the general overview of positively diagnosed patients. Rather, the Gomez-Lopez-Hernandez syndrome patient was moved to the diagnostic related research phase of our study.

Findings with unknown clinical relevance

In 23.5% of the patients the diagnostic tests revealed an abnormal finding with unknown clinical relevance. The chromosomal aberrations with unknown significance

**Table 3** Monogenic defects identified by specific DNA diagnostic tests in diagnostic phase of the study.

Case number	Patient id	Sex	Phenotype	Gene/ Accession number	Mutation	Inheritance	Clinical relevance
1	08-0034	M	Sev. ID; D; GR; MC; CA; E	<i>ATRX</i> /NM_000489.3	p.Ser1772Phe	Maternal	Pathogenic (OMIM 301040, ATRX syndrome)
2	09-0006	F	Mod. ID; D; N	<i>EHMT1</i> /NM_024757.3	c.3087+1G>T in intron 20 (dss)	<i>De novo</i>	Pathogenic (OMIM 610253, Kleefstra syndrome)
3	09-0008	M	Mild ID; D; N	<i>FGFR3</i> /NM_00142.3.	p.Pro250Arg	NT	Pathogenic (OMIM 602849, Muenke syndrome)
4	09-0020	M	Sev. ID; N; E; Fa	<i>MECP2</i> /NM_004992.2	p.Ser411fs	Maternal	Pathogenic (OMIM 312750, Rett syndrome)
5	09-0026	M	Mod. ID; D; GR; CA	<i>MID1</i> /NM000381.3	IVS7/exon 8	NT	Pathogenic (OMIM 300000, Opitz GBBB syndrome)
6	09-0040	M	Sev.ID; D; MC	<i>TCF4</i> /NM_001083962.1	p.Arg580Gln	<i>De novo</i>	Pathogenic (OMIM 610954, Pitt-Hopkins syndrome)
7	09-0043	M	Sev. ID; D	<i>RAI1</i> /NM_030665.3	Variation intron 5	Maternal	Non pathogenic
8	09-0051	M	Sev.ID; D; GR; CA	<i>MCT8</i> /NM_006517.4	Synonymous	NT	Non pathogenic
9	09-0084	M	Sev. ID; E	<i>SCN1A</i> /NM_001202435.1	p.Ser1768Arg	<i>De novo</i>	Pathogenic (OMIM 607208, Dravet syndrome)
10	10-0001	M	Mild ID; N	<i>DMPK</i> /NM_001081562.1	> 150 (CTG)n repeats in 3' UTR	Maternal	Pathogenic (OMIM 160900, Myotonic Dystrophy)
11	10-0003	F	Sev.ID; D; N; E	<i>CDKL5</i> / NM_003159.2	splice site mutation intron 7	<i>De novo</i>	Pathogenic (OMIM 300672, Rett syndrome variant with infantile spasms)
12	10-0006	M	Sev. ID; N; E	<i>SCN1A</i> /NM_001202435.1	p.Glu1176fs	<i>De novo</i>	Pathogenic (OMIM 607208, Dravet Syndrome)
13	10-0018	M	Sev. ID; GR	<i>RAI1</i> /NM_030665.3	p.Pro242Leu	NT	Non pathogenic (found in healthy controls)
14	10-0054	F	Mod. ID; D; Fa	<i>RSK2</i> /NM_004586.2	Intronic, affects splicing, exon 5 is skipped. p.?	NT	Pathogenic (OMIM 303600, Coffin-Lowry syndrome)

NT= not tested. Sev./Mod./Mild ID: severe/moderate/mild ID; D: dysmorphism; MC: microcephaly; MaC: macrocephaly; (IU)GR: (intra-uterine) growth retardation/short stature; CA: congenital anomaly; Fa: ID in family; N: neurological features; E: epilepsy. Dss: donor splice site. IVS: intron intervening sequence

are summarized in **table 4**. Homozygous regions  $\geq 3$  Mb were arbitrarily put into this category in **table 1**, with the argument that these regions are candidate regions for the localization of autosomal recessive (AR) ID genes, but this is not necessarily the case. An overview of detected homozygous regions is shown in **table 5**.

**(Likely) non-pathogenic findings**

In about one third (34.2%) we detected abnormal findings that are unlikely to have an association with the ID phenotype. These (likely) non-pathogenic chromosomal CNVs are summarized in **table 6**. The majority of (likely) non-pathogenic findings resulted from the metabolic screen and included non-specific, benign, inconsistent and/or iatrogenic abnormal values of metabolites in urine or serum that did not have an apparent relation with the ID phenotype. For example, mildly increased levels of alanine due to pharmacotherapy with sodium valproate were frequently observed.

**Table 4** CNV's with unknown clinical relevance (N=16).

Case number	Patient ID	Phenotype	Sex	CNV	Chromosomal region	Start-End Mb positions (Hg19)	Size/Gene content	Inheritance	SNP array platform	References
1	09-0021	Sev.ID; D; GR; CA; N	F	gain	1q21.1	145.51-145.71	200 kb/~10 genes	Maternal	250k SNP array	<sup>18-20</sup> Multiple overlapping cases reported in Decipher
2	09-0007 <sup>a</sup>	Mod. ID; MC; GR	F	gain	2p12p11.2	81.09-84.69	3.6 Mb/3 genes, including <i>SUCLG1</i>	Unknown (parents not available)	250k SNP array	<i>SUCLG1</i> (OMIM 245400): involved in autosomal recessive mitochondrial DNA depletion syndrome
3	10-0008	Sev. ID; D; MC; N	F	Mosaic gain	2q34	209.42-210.45	1,03 Mb/2 genes, including <i>MAP2</i> (disrupted)	Unknown (parents not available)	250k SNP array	<i>MAP2</i> (OMIM 157130): microtubule associated protein, important in neurogenesis <sup>52</sup>
4	10-0023	Sev.ID; MC; GR	M	gain	3p14.1	65.90-66.79	991 kb/3 genes (1 disrupted)	Unknown, but 3 unaffected sibs do not carry this gain	250k SNP array	-
5	11-0035	Mod. ID; GR; N	F	gain	5q31.2	138.52-139.61	1 Mb/ > 10 genes	Unknown (parents not available)	250k SNP array	Overlapping gain in female girl with developmental delay in personal database (inheritance unknown)
6	11-0032	Sev. ID; D; GR; CA; E	M	gain	5q35.2	172.87-174.57	1.7Mb/ 7 genes (including <i>MSX2</i> )	Paternal	250k SNP array analysis	<sup>53, 54</sup>
7	10-0017	Mild familial ID (affected female sib)	M	gain	9p13.3	33.56-34.51	950 kb/ 15 genes (2 disrupted)	Paternal	250k SNP array	OMIM 604366: <i>DNAI1</i> (AR Kartagener syndrome) Decipher: Case number 251082 (inherited from normal parent)
8	10-0074	Mod. ID; D	F	gain	10p15.1	4.86-5.23	365 kb/ 6 genes (1 disrupted)	Maternal	250k SNP array	Decipher: Case number 255355
9	11-0029	Mild ID; D	F	gain	14q32.11	89.10-89.45	350 kb / 2 genes (1 disrupted)	Maternal	250k SNP array	-
10	10-0068	Mild ID; D; MC	M	gain	15q26.3	100.00-100.59	600 kb/ 5 genes (including <i>MEF2A</i> )	Maternal (learning difficulties)	250k SNP array	Decipher: Case number 248272, 253919 (much larger, though overlapping)
11	09-0069	Mild ID; CA; N; E	M	gain	17q25.3	80.87-81.01	180 kb/2 genes	Unknown (father not available)	250k SNP array	-



Table 4 Continued.

Case number	Patient ID	Phenotype	Sex	CNV	Chromosomal region	Start-End Mb positions (Hg19)	Size/Gene content	Inheritance	SNP array platform	References
12	11-0022 <sup>*b</sup>	Mod. ID; CA	M	gain	18q12.2q13	37.19-37.50	320 kb/2 genes (disrupted)	Paternal	250k SNP array	Decipher: Case number 251555 (this patient has in addition another small gain of unknown significance)
13	10-0046	Mod. ID; D; CA	M	gain	20p12.2	9.73-9.88	150 kb/1 gene ( <i>PAK7</i> disrupted)	Unknown (parents not available)	2.7M array	Internal database: in 2 patients with MCA, in one inherited from parent. In other unknown inheritance. <i>PAK7</i> <sup>°</sup> may have important function in brain <sup>55</sup>
14	10-0098	Sev. ID; GR; MC; CA	M	loss	22q11.21q11.22	21.98-22.98	1 Mb/14 genes	Unknown (parents not available)	250k SNP array	<sup>46, 56</sup> Decipher: Case numbers: 250737, 4625, 2748
15	08-0010	Sev.ID; D; GR; CA; E	F	gain	22q11.23	25.08-25.23	150kb/2 genes	Unknown (father not available)	250k SNP array	-
16	08-0031	Mod. ID; N	M	gain	22q11.23	23.68-24.98	1.3 Mb/~25 genes	Maternal	250k SNP array	<sup>47</sup> Decipher: Case number 251348

NT= not tested. Sev./Mod./Mild ID: severe/moderate/mild ID; D: dysmorphism; MC: microcephaly; MaC: macrocephaly; (IU)GR: (intra-uterine) growth retardation/short stature; CA: congenital anomaly; Fa: ID in family; N: neurological features; E: epilepsy

<sup>\*a</sup> Based on metabolic investigations, this patients was diagnosed with a mitochondrial disorder. It is unknown whether the gain with proximal breakpoint through the gene *SUCLG1* has a causal role

<sup>\*b</sup> This patients has a (likely) pathogenic CNV as well (see **Table 2**)

<sup>\*c</sup> *PAK7* is alternatively called *PAK5*, which may be confusing

Classifications of findings in each individual patient in this cohort were based on careful consideration of the full picture of the criteria for assessment of clinical relevance as mentioned in **Chapter 2, Table 4**. None of the criteria was absolute and each criterion was considered in perspective to the other criteria, the currently available information in literature and databases, and the phenotype of the patient. Microdeletions and microduplications that can be considered as a strong risk factor for ID but have sporadically been reported in healthy controls or are inherited, such as 15q13 microdeletions and duplications, were either classified as (likely) pathogenic or as of unknown clinical significance depending on the phenotype of the respective patient.

6.2 Diagnostic related research phase

Fifty of the 191 (26.2%) patients in whom the diagnostic phase of the study did not reveal a molecular diagnosis were included in further studies in the second, diagnostic related research phase of the study. Various subgroups of undiagnosed patients were considered for further analyses, mainly by various next generation sequencing (NGS) approaches (in 47 patients), including 1) patients with sporadic ID, an IQ<50 and availability of both parental samples, 2) patients with familial ID, compatible with an X-linked (XL) and/or autosomal recessive (AR) pedigree, and availability of DNA samples of a sufficient number of affected and non-affected family members. Patients with genomic variants of unknown clinical significance (except for homozygous regions) were excluded from further NGS studies (see also **chapter 2**).

**Table 5** Homozygous regions detected (N=21).

Case number	Patient id	Familial ID	Chromosomal region	Size	Known ID associated genes	Consanguinity	Further evaluation
1	08-0015	Yes, but NA	Multiple	More and larger homozygous regions. In total 135 Mb	Multiple	Likely	No
2	08-0032	Yes, but NA	Multiple	More and larger homozygous regions	Multiple	Likely	No
3	08-0035	Yes	14q12	4.9 Mb	<i>FOXG1</i> (OMIM 164874)	Yes	Targeted NGS (including <i>FOXG1</i> ), followed by WES
4	09-0029	No	Multiple	More and larger homozygous regions	Multiple	Likely	No
5	09-0036	Unknown <sup>a</sup>	19p13.12, in overlap with milder affected brother	6.2 Mb	<i>CC2D1A</i> (OMIM 610055)	No	Sequencing <i>CC2D1A</i>
6	09-0039	Yes, but NA	17p12p11.2	8,8 Mb	6 (1 AD, 5 AR) <i>ALDH3A2</i> (OMIM 609523) <i>B9D1</i> (OMIM 614144) <i>ATPAF2</i> (OMIM 608918) <i>RAI1</i> (OMIM 607642) <i>TTC19</i> (OMIM 613814) <i>COX10</i> (OMIM 602125)	No	Clinical: phenotype does not fit with involvement of these genes Metabolic screen revealed no indication for mitochondrial disorder ( <i>ATPF2</i> , <i>TTC19</i> , <i>COX10</i> )
7	09-0050	No	Multiple, including large on chromosomes 1 and 9	18,1 Mb 12,9 Mb	Multiple	Yes	Trio based WES
8	09-0058	No	5q33.1q34	14 Mb	Multiple (>50 genes)	No	Trio based WES
9	09-0061	Yes	Multiple, some overlapping	More and larger homozygous regions	Multiple	Yes	WES
10	10-0024	No	5qter and multiple small	8 Mb	Multiple	Possibly	No
11	10-0032	No	2q33.1q34 8q22.1q22.3 8q23.1q24.11	10,5 Mb 7,4 Mb 11,7 Mb	7 mitochondrial disease genes <i>MKS3</i> (OMIM 607361) <i>COH2</i> (OMIM 216550) <i>TRPS1</i> (OMIM 604386)	Unknown	Metabolic screen revealed metabolic defects that might be indicative for a mitochondrial disorder. Further evaluation was not possible
12	10-0034	Yes	5q14.3q21.1	11,7 Mb (~40 genes)	1 AD ID gene: <i>MEF2C</i> (OMIM 600662)	No	No
13	10-0056	Yes	Multiple, some overlapping in affected sister	More and larger homozygous regions		Yes	WES
14	10-0076	Yes	3p21.31p21.1	8 Mb	7, mostly AR metabolic disorders	No	Metabolic screen was not indicative for metabolic disorder WES
15	10-0078	No	Diverse	More and larger homozygous regions	Multiple	Yes	No
16	10-0094	No	1q23.3q25.1	12,7 Mb	<i>TMCO1</i> (OMIM 614123) <i>DARS2</i> (OMIM 610956)	No	Clinical: phenotype does not fit with involvement of these genes

Table 5 Continued.

Case number	Patient id	Familial ID	Chromosomal region	Size	Known ID associated genes	Consanguinity	Further evaluation
17	10-0104	Yes	1p13.3p12 (overlapping with homozygous region in two affected sisters)	12,4 Mb	ALX3 (OMIM 606014) TSHB (OMIM 188540)	Yes	Clinical: phenotype does not fit with involvement of these genes
18	11-0009	No	4q26	4.3 Mb	PRSS12 (OMIM 606709)	No	Sequencing of PRSS12
19	11-0012	Yes , NA	Diverse	More and larger homozygous regions	Several	Yes	No
20	11-0019	No	Diverse	More and larger homozygous regions (in total 5% genome)	Several	Yes	No
21	11-0037	No	Diverse	More and larger homozygous regions	Several	Likely	No

NA: not available \*a cause of ID might be of different origin

Homozygosity mapping

Families of three patients with familial ID and who carried a significant homozygous region (table 5) were further analyzed by homozygosity mapping to assess the relevance of the homozygous region. In cases that all affected individuals in one family shared the same homozygous region, candidate genes in the overlapping homozygous regions were selected for Sanger sequencing. No mutations were found (see table 5).

WES in patients with sporadic ID and suspected autosomal dominant (AD) inheritance

Thirty-eight patients with likely sporadic autosomal dominant (AD) ID were included in a larger project applying a family based whole exome sequencing (WES) approach in 100 patient-parent trios to identify *de novo* mutations causing severe ID (see chapter 5.2).<sup>7</sup> In two patients with a clinically recognizable syndrome WES was applied as well. They were not included in the 100 trios project. In 22 (55%) one or more *de novo* mutations (confirmed by Sanger sequencing) were identified. Family based WES in the total of 40 patients has led to the identification of 14 (35%) (likely) pathogenic *de novo* mutations leading to a pertinent or plausible diagnosis (see table 7). In the diagnostic related research phase of this study, mutations in known ID genes and novel recurrent ID genes were considered to be pathogenic beyond any doubt. *De novo* mutations in genes with a biological link to an ID pathway were considered to

be plausible novel ID genes and thus likely pathogenic. In three patients pathogenic mutations in known ID genes were found, including *PDHA* (Pyruvate dehydrogenase E1-alpha deficiency, OMIM 312170), *GRIN2A* (Epilepsy with neurodevelopmental defects, OMIM 613971) and *LRP2* (Donnai-Barrow syndrome, OMIM 222448). Donnai-Barrow syndrome is an AR syndromic form of ID. The patient with a *de novo* mutation in *LRP2* had in addition a rare paternally-inherited, predicted pathogenic variant. Analyses to show that the *de novo* event occurred on the maternal haplotype were inconclusive, but based on the phenotype of the patient this was considered to be causative (see chapter 5.2). This illustrates that other patients with a suspected AD cause of ID that are selected for a family based WES approach may have AR ID as well. However, in the series of 100 ID trios the analyses for AR causes did not reveal any additional AR causes. Pathogenic mutations in novel recurrent ID genes were detected in three patients and included mutations in *DYNC1H1* (see chapter 5.1)<sup>8</sup>, *GATAD2B* and *CTNNB1*. Patients with mutations in these genes showed overlap in phenotype, which further confirmed a causal relation of these genes with the observed ID phenotypes. Mutations in *DYNC1H1* and *GATAD2B* were identified in single patients from the cohort described in chapter 2 of this thesis, but were recurrently found in patients from other in house series. Mutations in *CTNNB1* were identified in two patients from this cohort. In one of them (A10-0040) the mutation was identified by family-based WES. The second patient was not included in family-based WES studies (because parental samples were not available), but was included in a series

**Table 6** (Likely) non-pathogenic CNVs (N=11).

Case number	Patient ID	Phenotype	Sex	CNV	Chromosomal region	Start-End Mb positions (Hg19)	Size/Gene content	Inheritance	Chromosomal analysis	References
1	08-0011	Sev. ID; GR; CA	F	gain	2q31.1	179,70-180,12	400 kb/2 genes	Inherited Segregation in non affected family members	250k SNP array	-
2	08-0021	Mod. ID; N	M	loss	1q41	215,94-216,12	180 kb/1 gene (USH2A, OMIM 608400)	Unknown (parents not available)	250k SNP array	-
3	08-0034 <sup>a</sup>	Sev. ID; D; GR; MC; CA; E	M	gain	6q11.1	61,97-62,72	753 kb/2 genes	Maternal (healthy mother)	250k SNP array	Decipher: Case numbers 250628, 257521
4	09-0019	Mild ID; D	M	loss	6q12	67,18-67,90	720 kb/no genes	Unknown (parents not available)	250k SNP array	-
5	09-0030	Mod. ID; D; GR; Fa	F	loss	5q23.1	119,86-199,99	130 kb/ 1 gene	Inherited Segregation in non affected sister	250k SNP array	-
6	09-0041	Sev. ID; D; MC; E	M	loss	6p25.1	4,26-4,46	200 kb/ no genes	Unknown (mother not available)	250k SNP array	DGV: Larger overlapping deletions in controls
7	09-0082	Sev.ID; CA; N; E	M	gain	14q13.2	35,38-35,83	450 kb/ 6 genes	Unknown (parents not available)	250k SNP array	Decipher: Case number 253275: larger (2.37 Mb) overlapping gain inherited from normal parent
8	09-0028 <sup>b</sup>	Mild ID; D; CA	M	gain	18q22.1	65.65-66.45	800 kb/2 genes	Paternal	250k SNP array	-
9	10-0018	Sev. ID; GR	M	gain	21q21.1	18,78-19,07	290 kb/4 genes	Maternal	250k SNP array	Decipher: Case numbers 257242, 253459 (no phenotype reported)
10	10-0052	Mod. ID; E	F	gain	Xq28	148,26-148.73	460 kb/ 6 genes	Unknown (parents not available)	250k SNP array	<sup>50</sup>
11	11-0021	Mod.ID; D; CA; N	M	gain	22q13.31	45,79-46,26	470 kb/ 4 genes	Maternal	250k SNP array	DGV : Slightly smaller overlapping CNVs in controls

DGV: Database of Genomic Variants; NT= not tested.

Sev./Mod./Mild ID: severe/moderate/mild ID; D: dysmorphism; MC: microcephaly; MaC: macrocephaly; (IU)GR: (intra-uterine) growth retardation/short stature; CA :congenital anomaly; Fa: ID in family; N: neurological features; E: epilepsy

<sup>a</sup> This patient was diagnosed with ATRX syndrome

<sup>b</sup> This patients has a second, pathogenic CNV (see **table 2**, number 17)

**Table 7** (Likely) pathogenic de novo mutations identified by WES in a total of 40 patients.

Case number	Patient id	Gene	Accession number	cDNA level	Protein level	PhyloP score	Grantham score	Comment
1	08-0002	KIAA0467	NM_015284	c.3208C>T	p.Arg1070X	1.2	1000	Plausible novel ID gene <sup>57, 58</sup>
2	08-0006	DYNC1H1	NM_001376.4	c.11465A>C	p.Glu1518Lys	6	56	Novel recurrent ID gene ( <b>chapter 5.2</b> ) <sup>8</sup>
3	09-0002	MLL3	NM_170606.2	c.4441C>T	p.Arg1481X	6.2	1000	Plausible novel ID gene <sup>59</sup>
4	09-0003	GRIN2A	NM_000833.3	c.1655C>G	p.Pro522Arg	5.4	103	Known ID gene <sup>60</sup> (OMIM 613971)
5	09-0023	PDHA	NM_000284.3	c.328delinsAGA	p.Pro110ArgfsX71	-	-	Known ID gene (OMIM 312170)
6	10-0010	ASH1L	NM_018489.2	c.2170G>T	p.Ala724Ser	1.5	99	Plausible novel ID gene. <i>ASH1L</i> is a histon methyltransferase (OMIM 607860). Other histon methyl transferase genes have been implicated in ID phenotypes, such as <i>EHMT1</i> <sup>61</sup>
7	09-0079	GATAD2B	NM_020699.2	c.1408C>T	p.Gln470X	-	-	Recurrent novel ID gene (see <b>chapter 5.2</b> ) <sup>7</sup>
8	10-0040	CTNNB1	NM_001904.3	c.1272_1275del	p.Ser425ThrfsX11	-	-	Recurrent novel ID gene (see <b>chapter 5.2</b> ) <sup>7</sup>
9	10-0065	PPP2R5D	NM_006245	c.157C>T	p.Pro53Ser	1.9	74	Plausible novel ID gene. <i>PPP2R5D</i> is highly expressed in adult brain and is implicated in control of cell growth and division (OMIM* 601646)
10	09-0034	KIF5C	NM_004522.1	c.709G>A	p.Glu237Lys	6.1	56	Plausible novel ID gene (see <b>chapter 5.3</b> )
11	09-0058	COL4A3BP	NM_001130105.1	c.797C>G	p.Ser266Cys	6.1	112	Plausible novel ID gene. Lethal phenotypic in zebrafish embryo <sup>62</sup> (OMIM 604677)
12	10-0022	LRP2*	NM_004525.2	c.12437del	p.Gly4146GlufsX2	-	-	Known AR ID gene (Donnai-Barrow syndrome; OMIM 222448)
13	10-0064	EEF1A2	NM_001958.2	c.208G>A	p.Gly70Ser	5.5	56	Plausible novel ID gene. Causes lethal, neurologic phenotype in mice <sup>63</sup> (OMIM 602959)
14	10-0108	MYT1L	NM_015025.2	c.2636+1G>A	p.? - Exon skipping	5.7	-	Possible novel ID gene <sup>13</sup>

\*This patient has in addition a rare inherited missense mutation in *LRP2*, which is predicted to be deleterious

that was screened for recurrent mutations in plausible novel ID genes that initially had been identified in patients included in family-based WES (see **chapter 5.2**).<sup>7</sup> Eight patients had *de novo* mutations in plausible novel ID genes based on a biological link to an ID pathway. Two of these eight genes (*MYT1L* and *KIF5C*) were previously reported to be involved in microdeletions associated with ID.<sup>9-13</sup> Though the deletions comprising these genes cause haploinsufficiency, it is not likely that the missense mutation in *KIF5C* affecting the motor domain of the KIF5C protein results in loss-of-function. The phenotypic effect of this mutation is likely to be caused by other mechanisms (see **chapter 5.3**). **Table 7** gives an overview of (likely) pathogenic *de novo* mutations identified by WES in patients with sporadic ID.

**Next generation sequencing (NGS) in familial cases of ID**

Seven patients with familial ID were included in NGS studies. In four patients with a suspected X-linked (XL) cause of ID, sequencing of the X-exome was performed followed by segregation analysis of the variants in family members. X-exome sequencing revealed a likely pathogenic mutation in one of those families. A mutation in the gene *KIF4A* (OMIM 300521) segregated with the phenotype in affected family members, comprising mild to moderate ID and epilepsy (see **chapter 5.3**).

In three patients with possible AR or XL ID, WES was performed. In one of the two families with a suspected AR mode of inheritance, an overlapping homozygous



region was previously detected by 250k SNP array analysis (see **table 5**), which served as a guide for the selection of potential causative DNA variants following WES. In 2 of the 3 families WES revealed a (likely) pathogenic mutation. In a family with two affected brothers (A10-0076) a missense (c.1658A>T (p.V553E)) mutation in the X-linked ID gene *OPHN1* was found. The phenotype of these brothers, comprising moderate ID, cerebellar hypoplasia, mild spasticity and mild facial dysmorphism was comparable to other patients with mutations in this gene (OMIM 300486). Interestingly, this is the first missense mutation reported, suggesting that the predicted amino acid substitution affects an important domain of the OPHN1 protein. In the second family (10-0104) comprising of three affected sisters a homozygous missense mutation (c.484G>A (p.Gly162Arg)), in a plausible novel ID gene (*SLC6A17*) was identified. This gene is located in the overlapping homozygous region detected in these patients. The mutation segregated with the phenotype, consisting of moderate-severe ID, behaviour problems, mild facial dysmorphism and a progressive tremor. The involved gene is specifically expressed in the neuronal synapse. The SLC6A17 protein is a member of the SLC6 family of transporter proteins for neurotransmitters, amino acids, and osmolytes like betaine, taurine, and creatine.<sup>14, 15</sup> The mutation predicts a substitution of a conserved amino acid in a transmembrane domain of SLC6A17, and therefore a causal relation with the phenotype is very likely.

(Likely) pathogenic mutations identified in familial ID are summarized in **table 8**.

Table 8 (Likely) pathogenic mutations in familial ID identified by X-exome sequencing or WES.									
Case number	Patient id	Approach	Gene	Accession number	cDNA level	Protein level	PhyloP score	Grantham score	Comments
1	10-0075	X-exome sequencing	<i>KIF4A</i>	NM_012310.4	c.1489-8_1490delins10	p.? skipping of exon15	0.98	?	See <b>chapter 5.3</b>
2	10-0076	WES	<i>OPHN1</i>	NM_002547.2	c.1658A>T	p.Val553Glu	2.3	121	Known ID gene (OMIM 300486)
3	10-0104	WES	<i>SLC6A17</i>	NM_001010898.2	c.484G>A	p.Gly162Arg	6.3	125	Member of the SLC6 family of transporter proteins for neurotransmitters, expression in neuronal synapse <sup>14, 15</sup>

### 6.3 Total yield of diagnostic phase and diagnostic related research phase

In the diagnostic phase 43 diagnoses were established, resulting in a diagnosis in 18.4% of the participants. In the diagnostic related research phase a pertinent or plausible diagnosis was established in 18 additional cases, including in 17 out of 47 (36.2%) patients that received NGS analyses. In one patient a recurrent mutation in the novel ID gene *CTNNB1* was found. This patient was included in a screening cohort for recurrent mutations in novel ID genes (see **chapter 5.2**). The total yield of (likely) molecular diagnoses of the first phase (18.4%) and of the NGS studies in second phase of the study in our cohort of individuals with so far unexplained ID sums up to 54.6% (18.4+36.2%). Because only 47 of the 191 patients (24.6%) who remained undiagnosed in the first phase of the study were selected for NGS studies in the second phase, this resulted in a diagnostic yield of 26.1% (61 out of 234) in the total cohort.

### 6.4 Comparison between patients *with* and *without* a diagnosis

**Table 9** shows a comparison of the phenotypic characteristics between patients *with* and patients *without* an established diagnosis. Only the presence of the feature *facial dysmorphism* differed significantly between the groups *with* and *without* a diagnosis. In the group of patients *with* a (likely) diagnosis a significant higher number of individuals had facial dysmorphism. There were no significant differences in distribution of gender, age and level of ID between the two groups.

**Table 9** Comparison of groups *with* (N=61) and *without* (N=173) an established (likely) diagnosis

Patient Characteristics (%)	Diagnosis +	Diagnosis -	P-value
<b>Sex<sup>#</sup></b>			p=0.514
Male	37 (60.7)	113 (65.3)	
Female	24 (39.3)	60 (34.7)	
<b>Mean age</b>	37.35 (15.28)	36.89 (SD 17.96)	p=0.858
<b>Age Group (y)<sup>#</sup></b>			p=0.380
0-18	8 (13.1)	32 (18.5)	
18-40	26 (42.6)	58 (33.5)	
>40	27 (44.3)	83 (48.0)	
<b>Level of ID<sup>#</sup></b>			p=0.658
Mild (IQ 50-70)	13 (21.3)	47 (27.2)	
Moderate (IQ 35-50)	17 (27.9)	43 (24.9)	
Severe (IQ <35)	31 (50.8)	83 (47.9)	
<b>Selection Criteria (HPO terms)<sup>a</sup></b>			
Short stature or overgrowth <sup>b</sup>	17 (27.9)	53 (30.6)	p=0.685
Microcephaly or macrocephaly <sup>c</sup>	17 (27.9)	47 (27.2)	p=0.916
Facial dysmorphism	39 (63.9)*	80 (46.2)*	p=0.017
Congenital abnormality (non-CNS)	23 (37.7)	51 (29.5)	p=0.235
Abnormality of the CNS	18 (29.5)	45 (26.0)	p=0.597
Familial ID or consanguinity	26 (42.6)	73 (42.2)	p=0.954
(Epilepsy)	24 (39.3)	69 (39.9)	p=0.941
<b>Number of positive Selection Criteria<sup>#</sup></b>			p=0.157
1	18 (29.5)	66 (38.2)	
2-3	31 (50.8)	90 (52.0)	
4-5	12 (19.7)	15 (8.7)	
>5	0 (0)	2 (1.1)	
<b>Total<sup>d</sup></b>	61 (26.1)	173 (73.9)	234 (100)

CNS=central nervous system

\*significant difference

<sup>#</sup>no significant difference in distribution between the diagnosis + and the diagnosis - groups

<sup>a</sup> several participants fulfilled more than 1 selection feature

<sup>b</sup> short stature: height  $\leq$  -2 SD; overgrowth height  $\geq$  +2 SD

<sup>c</sup> microcephaly: head circumference  $<$ -2 SD/ $<$ P2; macrocephaly: head circumference  $>$ +2 SD/ $>$ P98

<sup>d</sup> excluding participating affected family members (see familial cases)

(epilepsy): epilepsy was not a selection criterion

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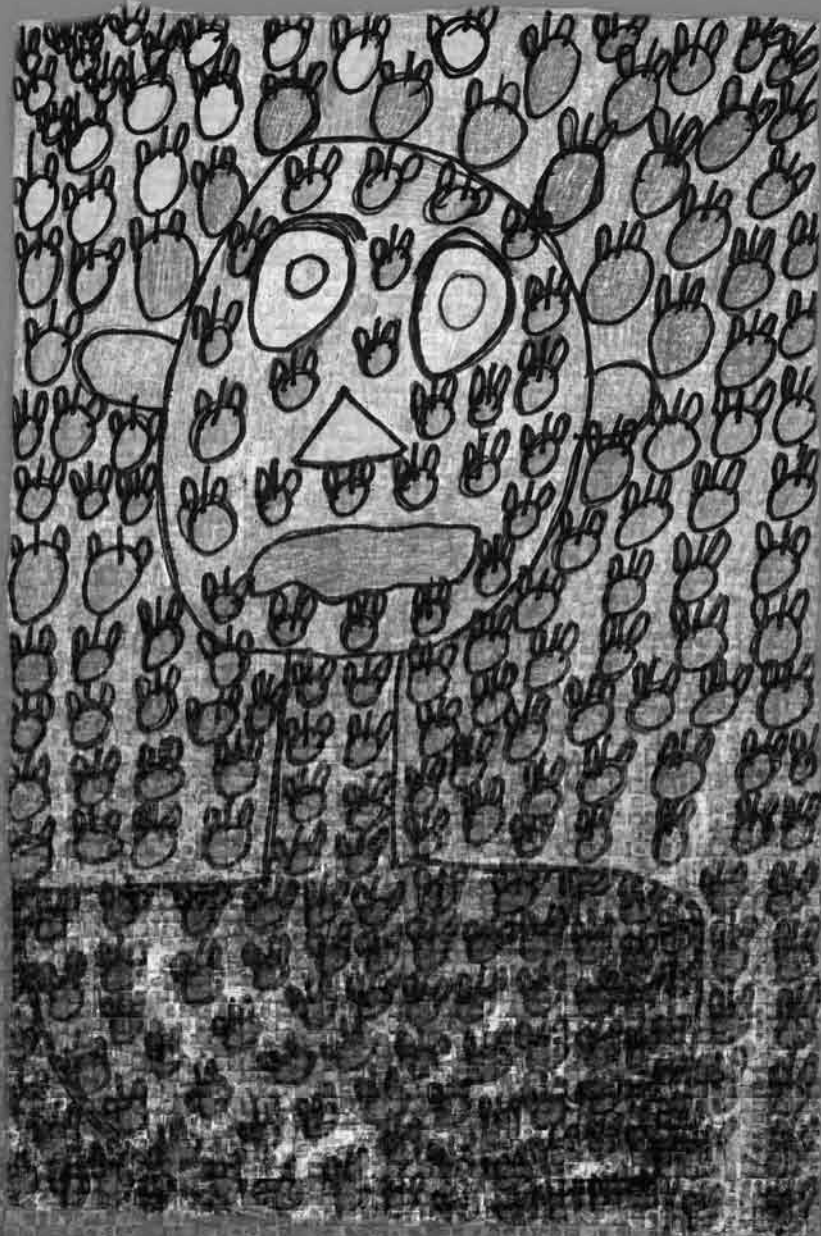
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# 7

## General discussion and future prospects

- 7.1 Yield of present-day genetic diagnostic assessment
- 7.2 Further phenotypic delineation of known ID syndromes
- 7.3 Identification and definition of novel ID syndromes
- 7.4 Challenges in clinical interpretation
- 7.5 Implementation in clinical practice: outpatient clinic  
"Zeldzaam" ("Unique")
- 7.6 Future prospects

The basic motivation of the studies described in this thesis is to increase the number of aetiological diagnoses in people with intellectual disability (ID), which is of major importance for affected individuals and their families. Before the era of the genome-wide array analysis and next generation sequencing (NGS) technologies, in 50%-70% of the people with ID the cause remained unexplained.<sup>1-6</sup> Moreover, many individuals living in residential settings of health care providers for people with ID, especially adults, have not received a present-day genetic diagnostic assessment.<sup>7</sup> Therefore, our studies focused on this large group of mainly adult individuals with unexplained ID (see **chapter 2**).

## 7.1 Yield of present-day genetic diagnostic assessment

### This study

This study was divided into two phases, reflecting the progress in genetic diagnostic technologies during the course of this project. During the first phase, referred to as the diagnostic phase of the study, we applied genetic diagnostic technologies that are now routinely used in clinical genetic practice. Patients included in our study cohort, of whom the majority were adults, had not yet received such a present-day genetic diagnostic assessment. Forty-three patients (18.4%) were diagnosed in the diagnostic phase of the study. Genome-wide array studies accounted for the major part of diagnoses (12.4%), which is in line with the results of previous studies assessing the diagnostic yield of genome-wide array analysis<sup>9-10</sup> and with the prediction of Baker *et al.*<sup>7</sup> that in 8-12% of the adults with idiopathic ID genome-wide array analysis would reveal a diagnosis. DNA diagnostic testing of specific genes based on clinical evaluation led to the diagnosis in 4.7% of the patients. Most diagnoses comprised ID syndromes for which the causative genes were identified recently, such as Pitt-Hopkins and Kleeftstra syndrome.<sup>11, 12</sup> This illustrates that clinical investigations in the light of present-day knowledge are a powerful tool in diagnostics of ID and underlines the value of periodic re-evaluation of patients with hitherto unexplained ID. This is facilitated by documentation of collective expertise in medical literature and databases, such as the Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources (DECIPHER), the European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations (ECARUCA), and the Database of Genomic Variants (DGV). The yield of metabolic screening in this study was very low (1.3%). This is in line with previous studies that also showed a relative low yield of metabolic investigations and a high percentage of abnormalities of non-specific and unknown significance. Therefore, selective and targeted metabolic testing has been recommended.<sup>2, 13-15</sup>



This study confirms that unselected metabolic testing of individuals with unexplained ID has a very low yield. Therefore, it is advisable to reserve metabolic studies for patients with a clinical suspicion, such as marked hypotonia and coarse facial features.

It is important to realize that the aforementioned numbers are based on a cohort of patients selected for previously unexplained ID. Therefore, easily identifiable and highly recognizable syndromes, such as trisomy 21, large microscopically visible structural chromosomal aberrations, Fragile X syndrome and frequent metabolic disorders such as phenylketonuria are not represented in this cohort (except for microscopically visible chromosomal aberrations in a few patients that had not yet received microscopic chromosomal analysis).

The introduction of next generation sequencing techniques (NGS) in research offered a great promise for providing diagnoses in patients who remained undiagnosed in the first diagnostic phase of the study.<sup>16-24</sup> Therefore, in the second, diagnostic related research phase of the study, we selected 47 of these 191 (24.6%) unsolved patients for NGS studies. The majority of them had sporadic ID and were included in a large family based whole exome sequencing project (WES) (**chapter 5.2**). Seven patients represented probands from ID families compatible with X-linked (XL) or autosomal recessive (AR) inheritance pattern. All together, NGS studies revealed a likely diagnosis in 17/47 (36.2%) of the included patients (see **figure 1**).

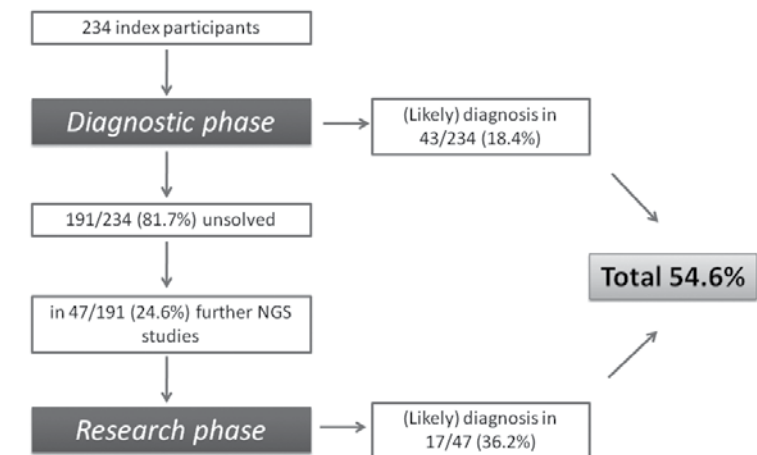
As described in **Chapter 6** the total yield of (likely) molecular diagnoses in individuals with so far unexplained ID sums up to 54.6% (18.4+36.2%). Forty-three individuals (18.4%) were diagnosed in the first diagnostic phase of the study. Due to the selection criteria we used, only about one quarter of the patients without a diagnosis in the first phase of the study were candidates for further NGS studies in the second phase of the study. For example, individuals with an IQ above 50, with an unknown copy number variation (CNV), with consanguineous parents, and/or individuals of whom one or both of the parents were not available, were excluded for the family based WES approach.

In total, in 61 participants a likely molecular diagnosis was established. In the total cohort of 234 participants this resulted in a diagnostic yield of 26.1%, however as explained above, only a small subset of the participants were included in further NGS studies. It is likely that more diagnoses would have been established without excluding these groups.

### Extrapolation of results

The impact of the application of present-day advanced genetic diagnostic technologies on the elucidation of genetic causes of unexplained ID in the total population of

**Figure 1** Yield in the diagnostic and diagnostic related research phase



people with ID, might be roughly estimated by extrapolating our study results. We made some assumptions to calculate this estimation. Firstly, we assumed that at least 50% of the cases of ID remained unexplained before the genome-wide array analysis era.<sup>1-6</sup> Secondly, we consider it likely that our study cohort is composed of individuals from this group of 50% unexplained cases of ID. This assumption is supported by the fact that none of the participants had received genome-wide array analysis. Extrapolation of the yield in the diagnostic phase of the present study (18.4%) to the total population of people with ID would then result in a 9.2% (18.4\*0.5) increase in the total number of patients with explained ID to approximately 59% (9.2%+50%). Extrapolation of the yield of additional NGS studies in the diagnostic related research part of the study would lead to an additional 18.1% (36.2\*0.5) increase in diagnoses to more than 75%.

Several remarks should be made. First, our study cohort may not be representative for the total population of people with ID. The cohort as presented in **chapter 2**, is composed of a selected population. Because most patients were recruited from residential settings of service providers, adult patients and patients with a moderate-severe ID (IQ <50) were overrepresented. The overrepresentation of patients with an IQ <50 might have led to an increased yield, though we did not observe a significant difference in the distribution of the level of ID between the group of patients with and without a diagnosis (see **chapter 6, table 9**). Secondly, we used the selection criterion that one or more other features than just ID had to be present

(see **chapter 2, table 2**). Although these additional findings could be very subtle, patients with additional findings to ID are overrepresented in our cohort as well. Within our study population we only observed a significant difference in the presence of facial dysmorphisms between the groups with and without diagnosis. In the group with an established likely diagnosis the number of individuals with facial dysmorphisms was significantly higher (**chapter 6, table 9**). There was also no significant difference in the number of positive selection criteria. However, the differences and the numbers in each group might be too small to reach significance. In the second phase of the study only a subset of the patients was selected for NGS studies. Application of NGS in the total group of patients that remained undiagnosed in the diagnostic phase of the study, might have resulted in a lower diagnostic rate of NGS studies, because we have now excluded individuals with an IQ between 50 and 70 from family based exome sequencing.

Despite of these limitations, our results may give a reasonable indication of the promising yield of the present-day advanced and rapidly developing genetic diagnostic repertoire. Furthermore, the application of WES in an increasing number of patients with ID, as well as in healthy controls will shed more light on the clinical significance of hitherto unknown variants. Some variants currently classified as “significance unknown” may turn out to be either pathogenic or non-pathogenic when more WES data become available. This may lead to establishment of additional diagnoses in patients with variants of currently unknown clinical significance. Furthermore, recurrent findings in additional patient series and/or functional studies may further confirm likely causative mutations. Therefore, the diagnostic rate of exome sequencing will likely further increase in the near future.

## 7.2 Further phenotypic delineation of known ID syndromes

During this study we performed an extensive phenotypic description of all participating patients. This has resulted in the collection of a wealth of data on phenotypes of rare genetic disorders with ID. Moreover, the cohort we studied is unique, in the sense that more than 45% of the patients had an age above 40 years, and more than 80% were adults, while previous reports of rare genetic ID syndromes mainly involved children (see **chapter 2**). As a consequence, clinical characteristics including associated health and behaviour issues and recognizable facial features are generally well studied in children, but not in adults. Often little is known about evolution of phenotypes across life span. We have collected valuable data on progression of phenotypes in rare genetic ID syndromes. Information on health and social outcomes is necessary for adequate management and follow-up of patients

and enables careful counselling of family members regarding prognosis, natural course of the disease and life expectancy. **Chapter 4** includes a detailed up-to-date phenotypic and molecular overview of a large group of patients with Kleefstra syndrome of various ages (**chapter 4.1**) and a detailed phenotypic characterization of adults with Angelman- and Rett-like syndromes (**chapter 4.2**).

## 7.3 Identification and definition of novel ID syndromes

Since ID is an extremely heterogeneous disorder, it is expected that many underlying genetic defects are still to be elucidated. Therefore, one of the objectives of this study was to identify and define novel genetic syndromes with ID. In **chapter 3.2** the identification and definition of two novel microdeletion syndromes is described. By studying the genes in the shortest region of overlap, we identified candidate genes for the phenotype. The results of our studies in patients with 16q24.3 microdeletion syndromes (**chapter 3.2.1**) were subsequently further confirmed by others.<sup>25-27</sup> Recently, one of the candidate genes in this syndrome, *ANKRD11*, was shown to be involved in KBG syndrome (OMIM 148050).<sup>28</sup> Sirmaci *et al.*, identified in 7 patients from 5 families with KBG syndrome mutations in *ANKRD11* by WES. The patients with a microdeletion in chromosomal region 16q24.3 described by us and others showed indeed phenotypic similarities, but did not show the full clinical presentation of KBG syndrome.

**Chapter 3.2.2** describes the clinical and molecular characterization of the novel 1p21.3 microdeletion syndrome and the identification of the microRNA gene *MIR137* as likely contributor to the ID phenotype. This was supported by expression studies of downstream targets of *MicroRNA137* (*MIR137*) as well as by expression studies of *Mir137* in the mouse cortical synapse.

The identification of novel ID associated genes by NGS approaches is described in **chapter 5**. **Chapter 5.1** reports the identification and definition of a novel ID syndrome in a child and an adult patient. Their phenotypes included severe ID, signs of neuronal migration defects and motor problems. WES revealed in these two patients recurrent *de novo* mutations in the novel ID gene *dynein, cytoplasmic 1, heavy chain 1* (*DYNC1H1*) (OMIM 600112), which is also known in association with neurological phenotypes, including Charcot-Marie-Tooth disease and spinal muscular atrophy.<sup>29, 30</sup> Although these patients show some clinical overlap with the patients from our study, their phenotype is notoriously different, because in the patients from our study the cognitive phenotype is much more prominent, whereas in the patients with Charcot-Marie-Tooth disease and spinal muscular atrophy the neurological phenotype is most prominent. Future detection of additional variants in this gene in other patients may shed more light on the genotype-phenotype

correlations and may clarify these important differences in phenotype. Furthermore, this may lead to further broadening and characterization of the phenotypic spectrum. **Chapter 5.2** describes the identification of recurrent *de novo* mutations in two other newly identified ID genes, including *CTNNB1* and *GATAD2B*. Detailed phenotype-genotype comparisons may result in the definition of novel ID syndromes. **Chapter 5.2** also reports the identification of potential novel ID genes of which the causal role and impact on the phenotype should be further confirmed by recurrent findings in additional patients. As more sequence data become available after the implementation of NGS in diagnostics of ID, more recurrent mutations may be identified and may enable comparison and definition of corresponding phenotypes. With this, close collaboration between clinicians and laboratory specialists is of major importance. **Chapter 5.3** describes the identification of mutations in two plausible novel ID genes from the kinesin family group (*KIF* genes), *KIF4A* and *KIF5C*, in an X-linked family and a sporadic patient, respectively. Their ID phenotypes are detailed and functional studies provided further evidence for a causal role of these genes in ID. Previous reports that relate *KIF* genes to human ID phenotypes are rare. *KIF4A*, *KIF5C* and also *DYNC1H1* are all genes involved in the microtubule transport pathway. This suggests that the microtubule transport pathway may well be an evolving ID pathway.

## 7.4 Challenges in clinical interpretation

The technological advances in the elucidation of genetic causes of ID go along with the detection of a large number of variants that are difficult to interpret. This confronts us with the challenging task to determine the clinical relevance of these unknown variants. These uncertainties are a major challenge in the counselling of patients and their families. Prediction of prognosis and recurrence risk can be very difficult in these situations and may require identification of additional cases for detailed phenotype-genotype comparison, extensive family studies, and functional and biochemical analyses in animal or cellular models. Furthermore, prenatal testing can only be considered when the assessment of clinical relevance is sufficiently confident. In addition to unknown variants, an increased risk of incidental findings that are not related to ID but have medical implications for individuals with ID and/or their parents may also complicate counselling and can give rise to ethical issues.<sup>22, 31</sup>

In the past several years the wide application of genome-wide array analysis in research and diagnostics of ID has provided and continues to provide a wealth of data on recurrent genomic CNVs, both in patients and in control individuals.

Collection of these molecular data in association with corresponding phenotypes in central databases, such as DECIPHER, ECARUCA and DGV importantly contributes to the assessment of clinical relevance of these CNVs.<sup>32-34</sup> Nevertheless, the clinical significance of many CNVs cannot be clarified at the moment, as is illustrated by the detection of CNVs of unknown clinical significance in 6.8% of the patients in the present cohort study (**chapter 6**).

**Chapter 3.2.3** describes the interpretation of clinical relevance of X-chromosomal copy number variations (X-CNVs) detected by genome-wide array analysis in a large cohort of patients who were referred to our diagnostic centre for the evaluation of ID or other cognitive disorders. This provides a resource for clinicians and laboratory specialist for the interpretation of X-CNVs that are picked up by genome wide array platforms in a routine diagnostic setting.

Likewise the genome-wide array analysis studies, the application of WES in this study revealed several genetic variations with an unclear pathogenicity, as reported in **chapter 5.2** and **chapter 6**. In analogy with the developments after the introduction of genome-wide array analysis, future large scale application of NGS technologies both in research and diagnostics may clarify the pathogenicity of several currently unknown or ambiguous variants. This highlights the importance of storage of molecular data in combination with detailed phenotypic data in central databases.<sup>31, 35</sup> More and more recurrent variants in patients and controls will be identified. Phenotype-genotype studies of these recurrent variants will shed more light on hitherto unknown variants.

## 7.5 Implementation in clinical practice: Outpatient clinic “Zeldzaam” (“Unique”)

Genetic causes of ID are very heterogeneous. Therefore, each single genetic disorder with ID is mostly very rare. As a consequence, available information on clinical presentation, including associated health and behaviour problems, prognosis and course of life, is scarce. Yet, this information is very important in care and counselling of people with ID and their families. The detailed clinical information of the participating, mainly adult individuals with ID collected during this study is thus highly valuable. It importantly contributes to the insight and knowledge of rare genetic disorders with ID and facilitates care and counselling of affected individuals and their families. Several previous reports have shown that identification of the underlying cause of ID contributes to specific management of patients with rare genetic causes of ID. Studies of cognitive behavioural profiles may guide specific

behavioural and pharmacological interventions. Several studies have reported specific behaviour profiles in certain ID syndromes. It was shown that patients with Kleefstra syndrome might develop a regressive behavioural phenotype at adolescent/adult age of which clinicians and care-takers should be aware of. This is described in **chapter 4.1.1** and also in previous publications from our group.<sup>36, 37</sup> During this study we diagnosed two brothers with Phelan-McDermid syndrome (22q13 deletion syndrome). Psychiatric evaluation of these brothers showed that in addition to delayed speech and perseverations with autistic-like features, the psychopathological phenotype of Phelan-McDermid syndrome may also comprise atypical bipolar disorders. The latter may have implications for the treatment regime. As the symptoms of these patients remitted under maintenance therapy with a mood stabilizer, compounds from this class may be considered in the treatment of fluctuating challenging behaviours in patients with ID and, more specifically, those with Phelan-McDermid syndrome in whom an (atypical) bipolar disorder may be present.<sup>38</sup> Previous studies in adults with Prader-Willi syndrome highlighted medical, psychiatric and behavioural issues that are important for the general management of patients with Prader-Willi syndrome.<sup>39-41</sup> Catarino and others showed that establishment of the diagnosis in adult patients with *SCN1A* mutations enabled a specific anti-epileptic treatment strategy leading to better seizure control, improvement of cognitive functioning and quality of life.<sup>42</sup> In an adult female diagnosed during this study with a deletion in the chromosomal region 3q24-q25.4, an associated hypogonadotrophic hypogonadism was observed, for which she was subsequently treated with bisphosphonates to prevent osteoporotic complications, which can be very disabling (**chapter 3.1.1**).

For this study we established in collaboration with the department of primary care, chair "Health care for people with ID", a multidisciplinary outpatient clinic (see **chapter 2**). After completion of the clinical evaluations in the patients from the study cohort, this outpatient clinic continued under the name "Zeldzaam" (or "Unique" in English). Outpatient clinic "Zeldzaam" aims to implement the acquired knowledge and insights on rare genetic disorders with ID in clinical practice. Moreover, the systematic follow-up of patients in this multidisciplinary setting that bundles the expertise of the clinical geneticist and the specialist ID physician, facilitates a further increase of our experience, provides additional data on medical and psychiatric comorbidity and may generate new research questions as well.

## 7.6 Future prospects

With the introduction of WES in the diagnostics of ID, extensive phenotyping will become increasingly less important in guiding diagnostics, and the success of identification of clinical recognizable ID syndromes will no longer be highly dependent on clinical expertise on syndrome recognition.<sup>31</sup> However, this does not implicate that phenotyping is no longer important, but rather means that the diagnostic approach will shift from a "phenotype first" to a "genotype first" approach (reverse phenotyping). In the near future, mapping of the genomic profile of individuals with ID may be the first step in the diagnostic process. Subsequent clinical investigations will be essential in the interpretation of detected genomic variants and to correlate these to the clinical presentation of the patient. Moreover, in-depth clinical investigations, including standardized psychometric cognitive testing, physical examinations, and longitudinal investigations during follow-up will stay beyond dispute for management and insight in prognosis of individuals with a specific genetic defect. The results of such studies derived from patient cohorts sharing similar molecular defects, and phenotypic features, will be mandatory for future trials in which the phenotypic effects of drugs will be assessed in clinical trials. As a consequence, the value of detailed clinical characterization of genetic disorders with ID in counselling and care of patients and their families will become increasingly important.

The reverse phenotyping approach may have several advantages in the care of patients with ID and their families. Incriminating and invasive diagnostic tests -such as lumbar puncture-, wrong diagnoses and delay in diagnosis may be prevented. Instead, identification of the underlying genetic defect in an early phase enables personalized medicine and specific management by anticipation on associated comorbidity and behavioural problems from the beginning.

Another consequence of the "genotype first" approach will be a broadening of the clinical spectrum of known ID syndromes, since genetic defects may be detected in patients with nonspecific phenotypes who would not have been selected for specific genetic tests on clinical grounds.

Mutations outside the coding regions that cause ID remain to be defined. It is expected that the application of WES techniques in research and diagnostics of ID will be rapidly followed-up by whole *genome* sequencing (WGS).<sup>31</sup> This will enable the detection of disease causing variants in non-coding regulatory sequences, such as intronic variants, as well. These variants may lead to altered expression of genes and elucidate novel epigenetic ID causing mechanisms. Epigenetic mechanisms have already been shown to play a role in several ID syndromes.<sup>43</sup>

In analogy to the application of genome-wide chromosomal analysis in diagnostics of ID, the introduction of WES and WGS is expected to reveal genetic variants that are more likely risk factors or contributive factors to ID, rather than being *the* cause. Such variants may be implicated in digenic or oligogenic causes of ID. Assessment of the clinical relevance of these variants and to explain how they interplay to cause ID, will be a major challenge in diagnostics and research of ID during the coming years.<sup>31</sup> The translation and communication of these complex issues to patients and their families will be a challenge for clinical geneticists.

The identification of genetic causes of ID is the first step towards the development of treatment strategies. Despite the fact that genetic causes of ID are extremely heterogeneous and over 400 genes have now been related to ID, it is supposed that this large number of genes converge onto a limited number of common underlying ID pathways. These pathways may be the target of future therapeutic interventions. Of course, it appears unreasonable to expect that in patient with severe ID normal intellect can be restored, but targeted interventions in ID related pathways might indeed improve cognitive, motor and social functioning and reduce degenerative aspects.<sup>44</sup> This may seem futuristic, but recent studies have shown that there are indeed opportunities for therapeutic strategies. Studies in animal models of Fragile X syndrome showed for example that metabotropic glutamate receptors 5 (mGluR5) antagonist and gamma amino-butyric acid (GABA) agonist can rescue various aspects of the Fragile X phenotype by targeting molecular pathways disrupted in Fragile X syndrome.<sup>45, 46</sup> Results of several clinical trials in humans have already been published and more studies are ongoing at the moment (reviewed by Hagerman *et al.*, 2012).<sup>45</sup> These studies showed promising results for pharmaco-therapeutical interventions that may not only improve behavioural aspects, but cognitive aspects as well.<sup>45</sup>

For several ID genes, such as *FMR1*, *MECP2*, and *EHMT1*, it has been shown that the mutations give rise not only to neurodevelopmental defects, but also acute effects that can be rescued at the adult stage. Studies in *EHMT* mutant *Drosophila melanogaster* showed that the learning and memory defects could be rescued when *EHMT* expression was restored in adult flies. In addition, conditional knockout of the Mouse *Ehmt1* gene at day 14 after birth gave rise to memory deficits, indicating that *Ehmt1* mutations have an effect beyond the developmental stage.<sup>47</sup>

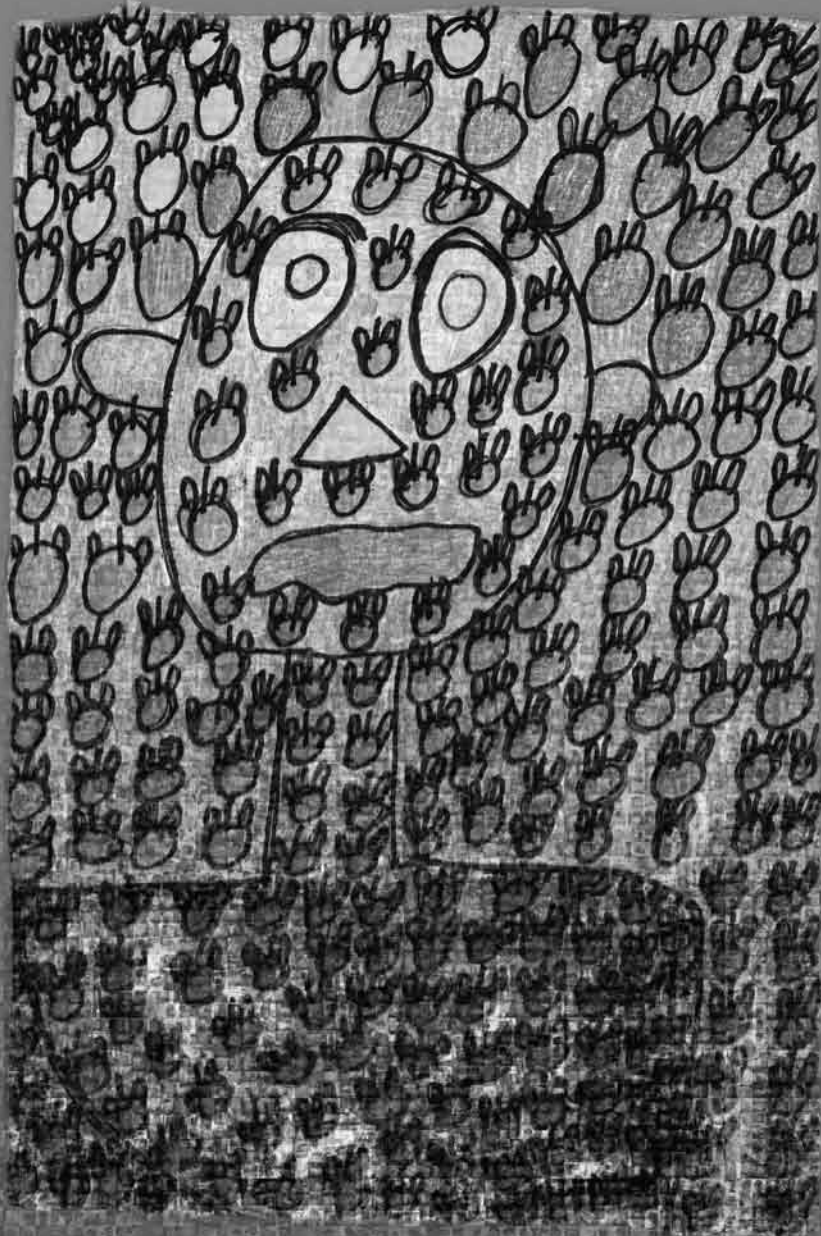
<sup>48</sup> These studies indicate that learning and memory are dynamic processes that might be rescued at least to some extent even when caused by genetic defects such as mutated *FMR1* and *EHMT*. This opens the window in understanding the mechanisms of ID and strongly emphasizes the need for further investigations on possible intervention strategies that might become available in future for some aspects of cognitive processes.

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Summary/Samenvatting

Dankwoord

List of publications

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Appendix (Color Figures)

## Summary

Intellectual disability (ID) has a major personal and social impact. It affects at least 120.000 people in the Netherlands and is associated with significant more psychiatric and physical comorbidity. Until recently, the cause of ID remained unknown in 50-70% of the affected individuals. Knowing the cause is of major importance in care and counselling of patients and families, and provides insight in comorbidity, associated behaviour problems, prognosis and recurrence risk. It precludes further unnecessary, often incriminating testing, and fruitless interventions, and instead enables specific anticipation on associated health and behavioral problems, since the extreme clinical and molecular heterogeneity goes along with diverse presentations and variable needs.

In recent years, advances in genetic technologies have provided great new opportunities for the elucidation of genetic causes of ID and the currently available genetic diagnostic repertoire is rapidly developing. In this study, state of the art genetic diagnostic technologies *and* a multidisciplinary clinical approach bundling the expertise of the clinical geneticist, specialist ID physician and other academic medical specialists were applied to identify genetic causes of ID. The aims of this study as defined in **chapter 1.6** included the identification of genetic causes of ID in individuals with previously unexplained ID, the detailed delineation of corresponding phenotypes, assessment of the diagnostic potential of the present-day genetic diagnostic repertoire and implementation of the gained insights in clinical practice.

In **chapter 2** the study organization and design, and the study cohort are described. This study was embedded in the Dutch research Consortium “Sterker op Eigen Benen/Stronger on Your Own Feet”, which is a collaboration between three large service providers for people with ID (*Pluyn*, *Dichterbij* and *Siza*) and the Radboud University Medical Centre in Nijmegen, the Netherlands. The 253 participants of the study, mainly adults with unexplained ID, were recruited from these service providers. The study comprised two phases. In the first phase, we performed in all participants clinical investigations followed by phenotype guided genetic diagnostic tests and standard tests that are currently routinely used in clinical genetic practice. About 25% of the participants undiagnosed in the first phase of the study, were included in further next generation sequencing (NGS) studies in the second phase of the study.

In **chapter 3** rarely reported and novel syndromes associated with microscopic and submicroscopic chromosomal aberrations are described. In **chapter 3.1.1**

three patients with overlapping interstitial 3q24q25 deletions were presented, who helped to delineate the critical region containing the gene(s) responsible for the Wisconsin syndrome phenotype. **Chapter 3.1.2** reports two patients with overlapping deletions in chromosomal region 8p12p21, revealing two candidate genes for congenital heart defects, which are sometimes observed in ID patients with a deletion overlapping this region. **Chapters 3.2.1** and **3.2.2** report clinical and molecular characteristics of two novel microdeletion syndromes, including four patients with 16q24.3 microdeletions (**3.2.1**) and five patients with 1p21.3 microdeletions (**3.2.2**). Study of the genes in the shortest regions of overlap revealed candidate genes involved in the ID phenotype, including *ZNF778* and *ANKRD11* in 16q24.3 microdeletion syndrome and *MIR137* in chromosome 1p21.3 microdeletions. **Chapter 3.2.3** deals with the clinical interpretation of X-chromosomal copy number variations (X-CNVs) identified by genome-wide array analysis.

In **chapter 4** detailed further phenotypic delineations of the well-known ID syndromes Kleefstra syndrome (**4.1**) and Angelman-and Rett like syndromes (**4.2**) are described, including the evolution of the phenotypes at adulthood.

In **chapter 5** the results of application of NGS studies in patients with sporadic ID and familial ID are reported, which has led to the identification of known and novel genes involved in ID, and the definition of novel ID syndromes. In addition several novel candidate ID genes were identified. **Chapter 5.1** describes two patients with *de novo* mutations in the novel ID gene *DYNC1H1*, presenting with a similar phenotype, comprising severe ID and variable neuronal migration defect, and thus representing a novel ID syndrome. **Chapter 5.2** reports the results of diagnostic exome sequencing using a family-based approach in patients with sporadic ID, which resulted in a definite diagnosis in 16%, mostly comprising *de novo* mutations. Amongst these were three novel ID genes with recurrent mutations in patients with similar phenotypes. In addition *de novo* mutations in several potential novel ID genes were identified.

In **chapter 5.3** the clinical characteristics of individuals with mutations in the kinesin superfamily (*KIF*) genes *KIF4A* and *KIF5C* are reported. Functional studies at the synapse of rat primary neurons further confirmed the involvement of *KIF4A* and *KIF5C* in the ID phenotypes of reported patients.

In **chapter 6** the total results of this study are described. In the first phase of the study a (likely) genetic diagnosis was established in 18.4%, comprising in 12.4% chromosomal aberrations, in 4.7% monogenic defects and in 1.3% metabolic disorders. NGS studies in the second phase of the study in sporadic and familial ID revealed a (likely) diagnosis in 36.2% of the included individuals, comprising

mutations in known ID genes, novel recurrent ID genes and plausible novel ID genes. The added yield of (likely) molecular diagnoses of both study phases was 54.6% (18.4% in first phase, 36.2% in NGS studies in the second phase). The specific findings are summarized in **tables 2-8 in chapter 6**.

**Chapter 7.1** reflects on the total results of this study. The total yield of likely diagnoses of this study sums up to more than 50% (54.6%). In addition these results are extrapolated to the total population of people with ID to illustrate the great promising opportunities of the present-day, rapidly developing genetic diagnostic repertoire and the impact on the total population of people with ID. This predicts that application of the currently available genetic diagnostic repertoire may lead to an increase in explained ID from 50% in the past to over 75%.

**Chapters 7.2-7.5** discuss the continuous importance of detailed phenotyping, though a shift from a "phenotype first" to a "genotype first" approach can be foreseen, which might have great advantages for patients. In **chapter 7.5** the implementation of study results in clinical practice are discussed, illustrated by our outpatient clinic "Unique (Zeldzaam)". Finally, **chapter 7.6** reflects on future prospects, such as the development of intervention strategies for the treatment of cognitive dysfunction targeting common underlying pathways involved in ID.

## Samenvatting

Verstandelijke beperking (VB) heeft een grote persoonlijke en maatschappelijke impact. In Nederland leven tenminste 120.000 mensen met een VB en zij hebben significant meer psychische en lichamelijke comorbiditeit. Tot voor kort bleef bij 50-70% van de mensen met een VB de oorzaak onduidelijk. Het weten van de oorzaak is van groot belang bij de counselling en de zorg voor mensen met een VB en hun familieleden. Het geeft bijvoorbeeld inzicht in comorbiditeit, geassocieerde gedragsproblemen, prognose en herhalingskans en kan een einde maken aan zinloze, en vaak belastende onderzoeken, en niet werkzame therapieën. In plaats daarvan kan gericht ingespeeld worden op specifieke geassocieerde gezondheids- en gedragsproblemen, omdat de grote klinische en moleculaire heterogeniteit van VB gepaard gaat met een verscheidenheid aan klinische presentaties en wisselende behoeften.

De afgelopen jaren hebben ontwikkelingen in genetische technologie geleid tot veelbelovende nieuwe mogelijkheden voor het opsporen van genetische oorzaken van VB en de beschikbare mogelijkheden voor genetische diagnostiek van VB ontwikkelen zich razendsnel. Tijdens het onderzoek beschreven in dit proefschrift werd enerzijds gebruik gemaakt van de meest moderne genetische technieken in de diagnostiek van VB en daarnaast van een multidisciplinaire klinische benadering waarbij de expertises van de klinisch geneticus, de arts voor verstandelijk gehandicapten (AVG) en andere academisch medisch specialisten gebundeld werden in de zoektocht naar genetische oorzaken van VB. De doelen van deze studie, zoals gedefinieerd in **hoofdstuk 1.6**, omvatten de identificatie van genetische oorzaken van VB in individuen met een tot dan toe onverklaarde VB, het gedetailleerd beschrijven van de bijbehorende klinische kenmerken (fenotypes), het vaststellen van de diagnostische mogelijkheden van het huidige arsenaal aan technieken voor genetische diagnostiek, en tot slot de implementatie van de verworven kennis in de klinische praktijk.

In **hoofdstuk 2** worden de organisatie en opzet van deze studie, en het onderzoekscohort beschreven. Deze studie vond plaats binnen het consortium “Sterker op Eigen Benen” dat een samenwerking is tussen drie grote instellingen voor mensen met een VB in Nederland (*Pluryn*, *Dichterbij* and *Siza*) en het Radboud Universitair Medisch Centrum in Nijmegen, Nederland. De 253 deelnemers, voornamelijk volwassenen met een onverklaarde VB, werden binnen deze instellingen geworven. De studie was verdeeld in twee fasen. In de eerste fase werden alle deelnemers klinisch onderzocht op onze multidisciplinaire polikliniek in aanwezigheid van een klinisch geneticus en een AVG. Aansluitend werd *op indicatie*



gerichte genetische diagnostiek aangevraagd. Tevens werd bij *alle* deelnemers de standaard genetische diagnostiek verricht die momenteel routinematig worden gebruikt in de klinisch genetische diagnostiek van VB. Ongeveer een kwart van de deelnemers zonder diagnose in de eerste fase van de studie werd geïnccludeerd in vervolgstudies in de tweede fase van de studie, waarin met name verschillende next generation sequencing (NGS) benaderingen werden toegepast.

In **hoofdstuk 3** worden zeldzame en nieuwe beelden die veroorzaakt worden door microscopische en submicroscopische chromosoomafwijkingen beschreven. In **hoofdstuk 3.1.1** worden drie patiënten met overlappende interstitiële deleties van de chromosomale regio 3q24q25 beschreven. Het klinisch en moleculair onderzoek bij deze patiënten heeft bijgedragen aan de afgrenzing van het kritische gebied dat de gen(en) bevat die verantwoordelijk worden gehouden voor het fenotype van het Wisconsin syndroom.

**Hoofdstuk 3.1.2** beschrijft twee patiënten met overlappende deleties van de chromosomale regio 8p12p21, op basis waarvan twee kandidaatgenen voor aangeboren hartafwijkingen geïdentificeerd werden, welke naast een VB bij deze patiënten vaker beschreven worden. **Hoofdstukken 3.2.1** en **3.2.2** beschrijven de klinische en moleculaire kenmerken van twee nieuwe microdeletie syndromen, vastgesteld bij respectievelijk vier patiënten met een microdeletie van chromosoom 16q24.3 (**3.2.1**), en vijf patiënten met een microdeletie van chromosoom 1p21.3 (**3.2.2**). Bestudering van de genen gelegen in de overlappende gebieden van deze microdeleties, bracht kandidaatgenen betrokken bij de fenotypes aan het licht, namelijk *ZNF778* en *ANKRD11* in het 16q24.3 microdeletie syndroom en *MIR137* in chromosoom 1p21.3 microdeleties. **Hoofdstuk 3.2.3** behandelt de klinische interpretatie van kopiënummervananderingen (CNVs) van het X-chromosoom, opgepikt met genomwijd submicroscopisch chromosomenonderzoek.

In **hoofdstuk 4** wordt het fenotypisch spectrum van bekende syndromen met VB, namelijk Kleefstra syndroom (**4.1**) en Angelman- en Rett-achtige syndromen (**4.2**) gedetailleerd besproken, inclusief de evolutie op volwassen leeftijd.

In **hoofdstuk 5** worden de resultaten van het toepassen van het recent in de diagnostiek geïntroduceerde NGS onderzoek bij sporadische patiënten met VB en bij patiënten met familiale VB beschreven. Dit heeft geleid tot het vaststellen van bekende en nieuwe genen betrokken bij VB, en de beschrijving van nieuwe syndromen met VB. Daarnaast werden verschillende nieuwe kandidaat genen voor VB gevonden. **Hoofdstuk 5.1** beschrijft twee patiënten met *de novo* mutaties in een nieuw gen geassocieerd met VB, genaamd *DYNC1H1*. Zij presenteerden zich met een vergelijkbaar fenotype dat gekenmerkt werd door een ernstige VB en een

wisselende presentatie van een neuronale migratiestoornis en vertegenwoordigen een nieuw syndroom met VB. In **hoofdstuk 5.2** worden de resultaten van exoom sequensen met een trio (ouders-kind) georiënteerde benadering in de diagnostiek van patiënten met een sporadische VB beschreven, hetgeen resulteerde in een zekere diagnose in 16%, voornamelijk tengevolge van *de novo* mutaties. Daarbij zaten drie nieuwe genen voor VB waarin additionele mutaties werden gevonden in niet-gerelateerde patiënten met een vergelijkbaar fenotype. Daarnaast werden diverse *de novo* mutaties gevonden in nieuwe kandidaat genen voor VB. In **hoofdstuk 5.3** worden de klinische kenmerken van individuen met een mutatie in de kinesine superfamilie (KIF) genen *KIF4A* en *KIF5C* beschreven. Functionele studies in de synapsen van primaire neuronen van ratten hebben geleid tot verdere bevestiging van betrokkenheid van deze genen bij het ontstaan van VB.

In **hoofdstuk 6** worden de totaal resultaten van dit onderzoek beschreven. In de eerste fase van het onderzoek werd een (waarschijnlijke) genetische diagnose vastgesteld bij 18.4%, betreffende in 12.4% een chromosoomafwijking, in 4.7% een monogene afwijking, en in 1.3% een metabole stoornis. NGS onderzoek in de tweede fase van de studie bij patiënten met een sporadische of een familiale vorm van VB bracht bij 36.2% van de geïnccludeerde individuen een (waarschijnlijke) moleculaire diagnose aan het licht. De totale opbrengst van (waarschijnlijke) moleculaire diagnoses in beide fasen van de studie komt daarmee op 54.6% (18.4% in de eerste fase plus 36.2% in de tweede fase). Specifieke bevindingen staan opgesomd in de **tabellen 2-8 van hoofdstuk 6**.

In **hoofdstuk 7.1** worden de totaalresultaten van deze studie besproken. Bij elkaar opgeteld is de opbrengst aan waarschijnlijke diagnoses van beide fasen van de studie meer dan 50% (54.6%). Daarnaast worden de resultaten behaald in dit studiecohort doorgetrokken naar de totale populatie van mensen met een VB om de enorme potentie van de huidige, zich snel ontwikkelende mogelijkheden voor genetische diagnostiek voor VB en de impact hiervan te illustreren. Dit laat zien dat het percentage mensen met een verklaarde VB, dat voorheen rond de 50% lag, door de toepassing van het huidig beschikbare genetisch diagnostisch arsenaal kan oplopen tot meer dan 75%.

Daarnaast wordt in **hoofdstuk 7 (7.2-7.5)** het blijvende belang van gedetailleerde fenotypering uitgebreid toegelicht, hoewel er een verschuiving wordt voorzien van een "fenotype eerst" naar een "genotype eerst" benadering, hetgeen mogelijk grote voordelen voor patiënten heeft. Daarnaast wordt in **hoofdstuk 7.5** de implementatie van deze onderzoeksresultaten in de klinische praktijk aan de hand van onze polikliniek Zeldzaam geïllustreerd. Tot slot wordt in **hoofdstuk 7.6** vooruit gekeken naar toekomstige ontwikkelingen, zoals de ontwikkeling van therapeutische

interventies voor VB die ingrijpen op gemeenschappelijke onderliggende pathways betrokken bij VB.

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\*shared first authorships

## Curriculum Vitae

Marjolein Hanna Willemsen werd op 28 februari 1981 geboren in Nijmegen, Nederland. In 1999 behaalde ze haar diploma Gymnasium aan het Dominicus College te Nijmegen. Aansluitend startte zij met de studie Geneeskunde aan de toen nog geheten Katholieke Universiteit Nijmegen (thans Radboud Universiteit Nijmegen). Haar Propedeuse (2000) en Doctoraal examen (2003) behaalde zij Cum Laude. In 2005 deed zij haar wetenschappelijk stage in het kader van haar studie Geneeskunde in Pécs, Hongarije, met als onderwerp "X-inactivatieonderzoek bij meisjes met pyruvaat dehydrogenase complex deficiëntie." In september 2005 begon zij na het behalen van haar Artsexamen als basisarts op de afdeling Kindergeneeskunde van het Rijnstate Ziekenhuis in Arnhem. Vanaf januari 2007 tot april 2008 werkte zij vervolgens als basisarts op de Neonatale Intensive Care Unit (NICU) van het Radboud Universitair Medisch Centrum in Nijmegen. Hierna maakte zij de overstap naar de afdeling Genetica waar zij in april 2008 begon als arts-onderzoeker op het onderzoeksproject "Diagnostiek en diagnostiek gerelateerde research van verstandelijke beperkingen met onbekende oorzaak." Gedurende dit project was zij nauw betrokken bij het oprichten van de multidisciplinaire polikliniek "Zeldzaam" voor diagnostiek en follow-up van mensen met zeldzame genetische oorzaken van verstandelijke beperking, die een samenwerking is tussen de afdelingen Genetica en Eerstelijns Geneeskunde, leerstoel "Geneeskunde voor mensen met verstandelijke beperkingen" van het Radboud Universitair Medisch Centrum in Nijmegen. Tijdens de duur van dit onderzoeksproject zag zij hier, samen met een klinisch geneticus en een arts voor verstandelijk gehandicapten (AVG), ruim 250 mensen met een onverklaarde verstandelijke beperking. Ook sinds de start van haar opleiding tot klinisch geneticus in januari 2012 blijft zij betrokken bij de verdere ontwikkeling van polikliniek "Zeldzaam". Op 26 juni 2012 ontving zij tijdens het jaarlijkse congres van de European Society of Human Genetics (ESHG) de *Isabelle Oberlé award* voor haar presentatie over haar onderzoek beschreven in dit proefschrift.



## Appendix (Color Figures)

### Chapter 3.1.1

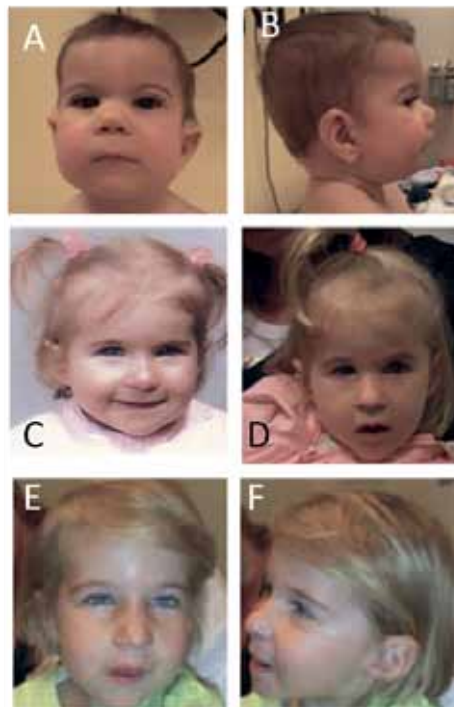
**Figure 1** Facial appearance of patient 1 at 60 years (b-c) and at younger adult age (a), of patient 2 at age 13 years (d-e) and of patient 3 at age 18 years (f-g) Note the coarse face, prominent nose, full lower lip, large mouth and unusually shaped, bushy eye brows.



### Chapter 3.1.2

**Figure 1** Photographs of two patients with a 8p12p21 deletion.

**A-B** Patient 1 at 9 months of age, showing long dark eye lashes, arched eyebrows, inverted epicanthal folds, a long and smooth philtrum, thin lips, downturned corners of the mouth and over folded upper helices of the ears. **C-F** Patient 2 at age of 12 months (C), 20 months (D) and 33 months (E-F), showing mild facial dysmorphic features, including thin upper lip, a full hooked nose and narrow palpebral fissures.



### Chapter 3.2.1

**Figure 1** Facial profiles of patients 1-4. **A-C**: Patient 1 at several ages from 6 years (A) to 22 years (B-C). **D-F**: Patient 2 at the age of 3 years (D) and at the age of 8 years (E-F). **G-H**: Patient 3 at the age of 7 years. **I-J**: Patient 4 at the age of 8 years and 10 months. Facial features comprise high forehead/frontal bossing and large ears (all patients), broad mouth, long smooth philtrum and pointed chin (patient 1-3). Note the change in facial phenotype in patient 1 at adult age, showing a long and oval face with full upturned nose, retrognathia and pronounced groove in the chin.



### Chapter 3.2.2

**Figure 1** Facial appearance of patients 1, 2, 4 and 5.

Figures **A-B** show patient 1 at age 42 years, figures **C-D** patient 2 at age 38 years, figures **E-F** patient 4 at 34 years and figures **G-H** patient 5 at age 18 years. Note the minor facial dysmorphic features, including long ears, deeply set eyes, full tip of the nose and thick lower lip.



### Chapter 4.1.1

**Figure 3** **A-V** Facial profiles of patients with a 9q34.3 deletion, including patient 1 at age 2 years (**A**) and 5 years (**B**), patient 2 at age 9 months (**C**), 2 years (**D**) and 6 years (**E**), patient 4 at age 5 years (**F, G**), patient 5 at age 11 years (**H, I**), patient 6 at age 1 year (**J**) and 2 years (**K**), patient 7 at age 9 months (**L**), patient 8 at different childhood ages (**M-O**), patient 10 at young childhood age (**P, Q**), patient 11 at age 3 years (**R, S**) and 5 years (**T, U**) and patient 12 at age 5 years (**V**). The highly recognizable facial features comprise hypertelorism, midface hypoplasia, prognathism, prominent eyebrows, cupid bow or tented upper lip and everted lower lip.



## Chapter 4.1.1

**Figure 4** A–W Facial appearance of patients with an EHMT1 mutation, including patient 17 at age 2 years (A, B), patient 18 at age 1 year (C) and 6 years (D, E), patient 19 at age 3 years (F, G), patient 20 at age 41 years (H, I), patient 21 at age 5 years (J, K), patient 24 at age 2 years (L, M) and 10 years (N), patient 25 at childhood age (O), during teenage (P), and age 32 years (Q, R), patient 26 at age 2 years (S) and age 10 years (T, U), patient 27 at age 11 years (V, W). Facial characteristics are similar to Kleefstra syndrome patients with a 9q34.3 deletion.



## Chapter 4.1.2

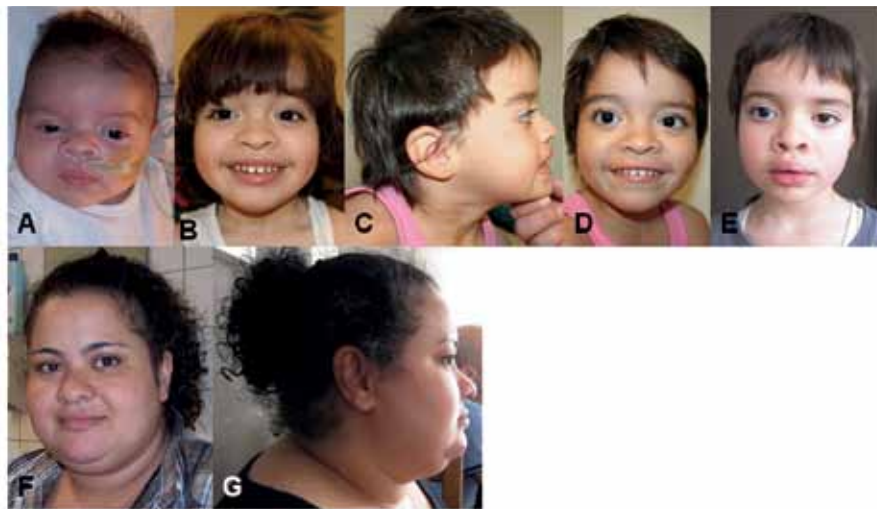
**Figure 1** Photograph of family 1. The proband (left) with his younger brother and his mother. Note the characteristic facial appearance of Kleefstra syndrome in the proband and the mild facial features in the mother. The younger brother shows marked hypotonia.





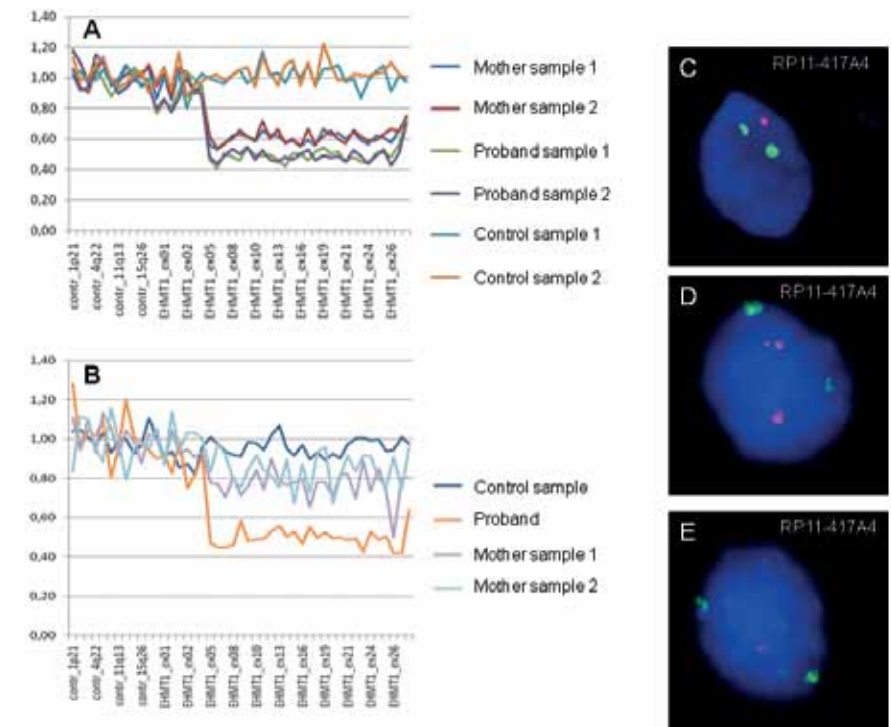
## Chapter 4.1.2

**Figure 2** Photographs of family 2. The proband at the age of 2 months (a), 3 years (b), 4 years and 6 months (c, d) and 5 years (e), and her mother (f, g). Note the hypertelorism, broad and depressed bridge of the nose, flared nostrils (a, b, d, e), mild midface hypoplasia (c) and broad mouth with full lips in the proband (b, d, e). The mother has marked obesity and minor dysmorphic features, including a hypoplastic midface, small palpebral fissures, a depressed nasal bridge and anteverted nares (f, g).



## Chapter 4.1.2

**Figure 4** Mosaic pattern detection in family 1 and 2. The results for 4 control probes and exons 1, 2, 5, 8, 10, 13, 16, 19, 21, 24 and 26 of the *EHMT1* gene are indicated. (a) and (b) indicate the results of the MLPA analysis in the proband and the mother of family 1, showing mosaicism for the 9q34.3 deletion in both blood lymphocytes (a) and fibroblasts (b) of the mother. Note that a lower percentage of the fibroblasts carries the 9q deletion as compared to the lymphocytes. (c)-(e) show the results of the FISH analysis on cells derived from buccal swabs in the proband and the mother of family 2. (c) shows only one red signal, indicating the deletion of probe RP11-417A4 (red color) in chromosome region 9q34.3 in the proband. (d) and (e) show mosaicism in the mother with a normal signal pattern for probe RP11-417A4 in (d) (two red signals) and only one red signal in (e), indicative for a loss in 9q34.4 in this cell. The centromere 9 probe is labeled green and shows a normal FISH pattern (two signals) in (c)-(e).





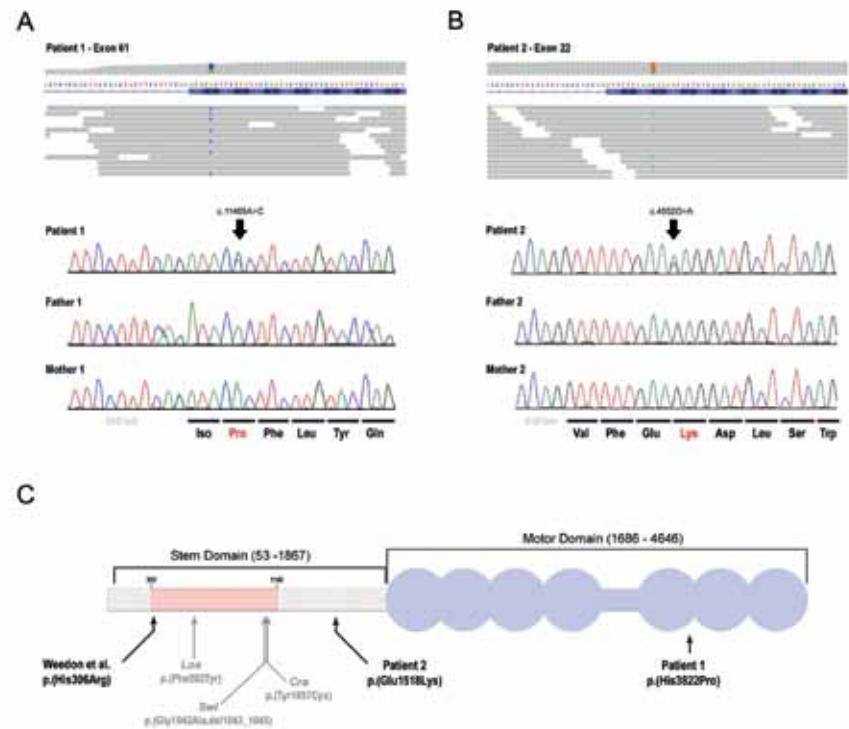
Chapter 4.2

**Figure 1** Clinical pictures, **A** and **B** show the typical facial features of Kleefstra syndrome in patient 1. Note the midface hypoplasia, arched eyebrows and prognathism. **C** and **D** show the typical facial appearance of Pitt Hopkins syndrome in patient 2. Note the coarse face with prognathism, broad mouth with down turned corners and large nose. **E-H** show patient 3 with a mutation in *MECP2* (E-F) and his carrier sister (G-H). I-J show patient 4 in whom we identified a mutation in *CDKL5*. Note her coarse face. K-N show patient 5 (K-L) and 6 (M-N) with Dravet syndrome. **O-P** show patient 7 with Phelan-McDermid syndrome. Note the long face and full lips. **Q-T** show the brother pair with Phelan-McDermid syndrome. Note the long face, prominent chin and large ears in both brothers.



Chapter 5.1

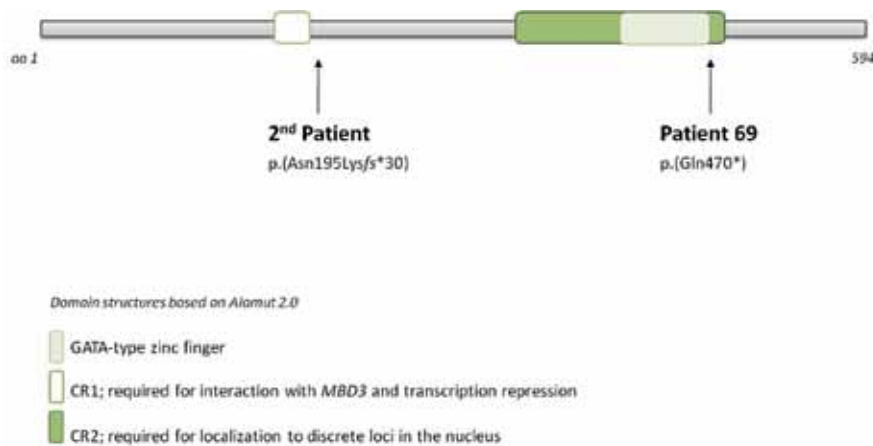
**Figure 2** Schematic representation of human DYNC1H1 and mutations in patient 1, 2 and the axonal (type 2) form of Charcot-Marie-Tooth (CMT2) disease family reported in Weedon *et al.*<sup>22</sup> together with the mutations of known mouse models.



Schematic representation of the mapped exome sequencing reads visualized using the *Integrative Genomics Viewer (IGV)* browser for patient 1 (panel A) and patient 2 (panel B), respectively. The upper part shows the per-base coverage, with coverage represented in gray indicating the wild-type base, whereas colored bases indicate the detection of variants. Also, a representation (part of) of exon 61 and exon 22 are provided for orientation. Individual sequence reads for patient 1 and patient 2 both show a heterozygous variant, which was followed up by Sanger sequencing, confirming the *de novo* occurrence in both patients. In panel (C), the DYNC1H1 protein is visualized according to Weedon *et al.* with the N-terminal region indicated by a gray horizontal bar, and the stem domain shown on above (amino acids 53-1867). The residues involving DYNC1H1 dimerisation (300-1140) are shown by a pink bar. The C-terminal motor domain (amino acids 1868-4646) is shown in purple colour, with the seven ATPase domains represented by circles and the stalk region by a horizontal bar. The equivalent positions of mutations in three mouse models, Loa, Swl, and Cra1, are shown below the representation. Note that the human protein contains two additional glycine residues at position 7 relative to mouse Dync1h1, that is numbering of equivalent residues in human DYNC1H1 is 2 higher than in mouse models. The three mutations reported in patients, including p.(His3822Pro) in patient 1, p.(Glu1518Lys) in patient 2, and p.(His306Arg) reported by Weedon *et al.* are indicated with arrows according to their relative positions of the functional domains of the DYNC1H1 protein.

Chapter 5.2

Figure 3 Schematic overview of GATAD2B mutations.

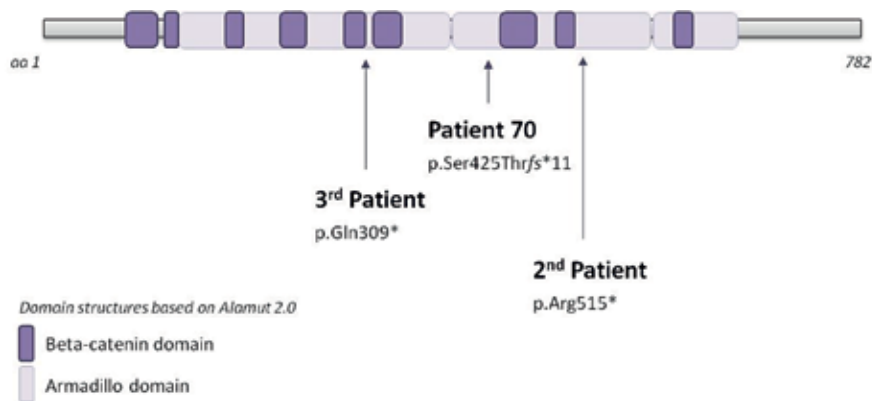


Schematic representation of GATAD2B, with its domain structure based on Alamut2.0. In addition, the mutations identified in this gene are indicated with their respective location at protein level. aa denotes amino acid number.

**Clinical comparison of patients with mutations in GATAD2B** – Two patients were shown to have a *de novo* mutation, p.(Gln470\*) and p.(Asn195Lysfs\*30) respectively, in the transcriptional repressor GATAD2B. Phenotypic comparison of these two patients was highly suggestive for a related genetic cause. They both had severe developmental delay with delayed motor milestones, only limited speech and overlapping facial features.

Chapter 5.2

Figure 4 Schematic overview of CTNNB1 mutations.



Schematic representation of CTNNB1, with its domain structure based on Alamut2.0. In addition, the mutations identified in this gene are indicated with their respective location at protein level. aa denotes amino acid number

**Clinical comparison of patients with mutations in CTNNB1** – Two patients were shown to have a *de novo* loss-of-function mutation in CTNNB1, including a p.(Arg515\*) nonsense mutation and a p.(Ser425Thrfs\*11) frameshift mutation. For a third patient, carrying a p.(Gln309\*) nonsense mutation, only maternal DNA was available for testing its segregation and was found to be absent. All patients presented with similar features comprising severe ID with absent or very limited speech, microcephaly and spasticity due to which the ability to walk was severely impaired.



